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APPEAL BRIEF Address to: Mail Stop Appeal Brief-Patents Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Application Number	10/723,955
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	First Named Inventor	Ruoping Chen
	Examiner	Ruixiang Li
	Group Art	1646
	Title: <i>Constitutively Activated Human G Protein Coupled Receptors</i>	

Sir:

This Brief is filed in support of Appellants' appeal from the Examiner's Rejection dated October 13, 2010. A Notice of Appeal was filed on November 15, 2010.

The Board of Appeals and Interferences has jurisdiction over this appeal pursuant to 35 U.S.C. §134.

Provided herewith is an authorization to charge the amount of \$270.00 to cover the fee required under 37 C.F.R. § 41.20(b)(2) for filing Appellant's Brief. In the unlikely event that the fee transmittal or other papers are separated from this document and/or other fees or relief are required, Appellants petition for such relief, including extensions of time, and authorize the Commissioner to charge any fees under 37 C.F.R. §§ 1.16, 1.17 and 1.21 which may be required by this paper, or to credit any overpayment, to deposit account number 50-0815, reference number AREN-007CON2.

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REAL PARTY IN INTEREST

The inventors named on this patent application assigned their entire rights to the invention to Arena Pharmaceuticals, Inc.

RELATED APPEALS AND INTERFERENCES

There are currently no other appeals or interferences known to Appellants, the undersigned Appellants' representative, or the assignee to whom the inventors assigned their rights in the instant case, which would directly affect or be directly affected by, or have a bearing on the Board's decision in the instant appeal.

STATUS OF CLAIMS

Claims 1-68 were canceled. Claims 69-88 are pending. Claims 69-88 are rejected and are appealed herein.

STATUS OF AMENDMENTS

No amendments to the claims were filed subsequent to issuance of the prior Office Action.

SUMMARY OF CLAIMED SUBJECT MATTER

The claims are directed to a method of screening for a compound that increases cAMP levels in peripheral blood leukocytes. The method comprises: (a) contacting a candidate compound with a G protein-coupled receptor (GPCR) that is present on the surface of a recombinant host cell or isolated membrane thereof, wherein the GPCR comprises an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:82 (page 9, line 8-13; page 13, lines 5-29; page 15 line 7 to page 17 line 3); and (b) determining that the candidate compound is an agonist of the

GPCR (page 6, lines 1-9; page 4 lines 30-31) and; wherein an agonist of the GPCR is capable of increasing cAMP levels in peripheral blood leukocytes (page 4 lines 15-21 and Figs. 2A, 2B, 4A, 4B, 5A, 5B and 6).

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

- I. Rejection of claims 69-88 under 35 U.S.C. § 101, first paragraph because the claimed invention allegedly is not supported by either a specific and substantial asserted utility or a well-established utility.
- II. Rejection of claims 69-88 under 35 U.S.C. § 112, first paragraph because the claimed invention allegedly is not supported by either a specific and substantial asserted utility or a well-established utility.

ARGUMENT

I. Claims 69-88 are rejected under 35 U.S.C. § 101 as allegedly being unsupported by a patentable utility. The Appellants respectfully traverse this rejection.

MPEP § 2107 provides guidelines for examination of applications for compliance with the utility requirement. According to MPEP 2107.II, a claimed invention is in compliance with §101 if it has a well-established utility or a utility that is asserted in the patent application. In both cases, the utility should be specific, substantial and credible.

An invention has a well-established utility if (i) a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention (e.g., properties or applications of a product or process), and (ii) the utility is specific, substantial, and credible. MPEP § 2107.II.A.3.

The claims are directed to a screening assay that uses a GPCR comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:82. SEQ ID NO:82 provides the amino acid sequence of human TDAG8.

The question is whether the rejected claims, which require the use of a polypeptide having at least 80% identity to TDAG8, have patentable utility.

Utility of the rejected claims is supported by the following facts:

- TDAG8 (SEQ ID NO:82) is preferentially expressed in organs that contain cells of the immune system, e.g., peripheral blood leukocytes, spleen and lymph node (see, e.g., Example 9 and Fig. 6).
- Activation of TDAG8 (e.g., by agonists such as ATP and ADP, as described in the subject application) leads to an increase in intracellular cAMP accumulation (see, e.g., Example 4 and Figs. 2A and 2B).
- Increased constitutive activity of TDAG8 leads to an increase in cAMP accumulation (see, e.g., Table G, Table I and Figs. 5A and 5B).
- Elevated cAMP accumulation in peripheral blood leukocytes is known to inhibit inflammation.¹
- The role of ATP and ADP in modulating inflammation is known.²

In view of the fact that: a) TDAG8 is preferentially expressed in organs containing immune cells; b) activation of TDAG8 (e.g., by agonists such as ATP and ADP, as described in the subject application, leads to an increase in intracellular cAMP accumulation; c) elevated cAMP accumulation in peripheral blood leukocytes inhibits inflammation and d) the role of ATP and ADP in modulating inflammation is known, it follows that the utility of TDAG8 would be

¹ See, e.g., Moore et al (Clin. Exp. Immunol. 1995 101:387-389; Naik (Eur. J. Pharmacology 1984 104: 253-259); Deporter et al (Br. J. Pharmac. 1979 65: 163-165); Deporter (Br. J. Pharmac. 1977 60: 205-207); and Bonta (Prostaglandins 1981 22 95-103. See References supplied in Evidence Appendix. References are of record.

² See, e.g., Brake et al (Chemistry and Biology 1996 3: 229-232); Cronstein (J. Appl. Physiol. 1994 76: 5-13); and Daval (Pharmacol. Ther. 1996 71: 325-335). References supplied in Evidence Appendix. References are of record.

readily apparent to one of skill in the art.

Specifically, increasing cAMP levels in peripheral blood leukocytes (PBLs) decreases the production of pro-inflammatory mediators, including platelet activating factor, γ -interferon and TNF, and increases the production of anti-inflammatory mediators, including IL-10.³ Since the claims are directed to a method of identifying compounds that increase cAMP levels in PBLs, one of skill in the art would immediately appreciate that the claims have a clear utility in identifying compounds that have anti-inflammatory activity.

The data provided in the instant application combined with what was known about the role of cAMP in leukocytes before the instant application was filed would allow one of skill in the art to immediately appreciate the utility of the claimed invention. As such, this rejection should be reversed.

In attempting to establish this rejection in the Office Action of October 13, 2010, the Examiner made several erroneous statements. These erroneous statements are addressed below.

Appellants' arguments are not invalid

In the Office Action, the Examiner argues that the Appellants' arguments are invalid, and that the Appellants are "purely guessing" about the role of TDAG8. Specifically, on page 4 of the Office Action the Examiner stated that "a poster presentation by some of the inventors of the instant application dated on 2000 (see IDS submitted on 08/30/2005)⁴, Applicants suggests that ATP binds TDAG8 and causes apoptosis of thymocytes. Now, Appellants argue that ATP

³ See, e.g., Eigler (J. Leukocyte Biology 1998 63: 101-107); Moore et al (Clin. Exp. Immunol. 1995 101: 387-389) and Benbernou (Immunology 1997 91: 361-368). These references were published before the priority date of the instant application.

⁴ Chen et al, "Identification of TDAG8 as a P2Y Purinergic Receptor", poster; February 26-March 3, 2000: Genome Tri Conference 2000, San Francisco, CA; poster abstract cited in the IDS submitted on August 30, 2005.

binds TDAG8 and mediating inflammation in peripheral blood leukocytes, clearly indicating that Applicants have no idea of what the functional activity of TDAG8 has and that Applicants are purely guessing about the functional role of TDAG8".

Anyone familiar with a scientific paper or poster presentation would know, the teaching of a first utility absent the concurrent teaching of a second utility cannot *a priori* be construed as a teaching against the second utility or that the first utility is the only utility. Specifically, a teaching that an agonist of TDAG8 can cause apoptosis of thymocytes does not *a priori* teach that an agonist of TDAG8 does not inhibit inflammation. In fact, those in the art at the time of filing would have understood that it was possible for a compound that causes apoptosis of thymocytes could also inhibit inflammation. Many compounds have several effects in the body.

In other words, the utility of TDAG8 in inhibiting inflammation discussed above is not inconsistent with what was known about mouse TDAG8, which is described on page 3 of the application. Specifically, page 3 of the specification states that TDAG8 is a T-cell death-associated receptor (page 3, lines 23-24) and that there is a strong correlation between apoptosis and TDAG8 (see page 3, line 28; see also Choi et al, provided as an Exhibit). Because T cells are a component of peripheral blood and dysregulation of T cell apoptosis is associated with inflammatory diseases (e.g., inflammatory bowel disease; see, e.g., Neurath et al Trends Immunol. 2001 22:21-6), TDAG8's role in inhibiting inflammation in PBLs is not incredible based on what was known in the art.

293 cells are an accepted model

On page 5 of the Office Action, the Examiner acknowledges that the instant application provides data showing that activation of TDAG8 by agonists such as ATP and ADP leads to an increase in intracellular cAMP accumulation. However, even given this data, the Examiner argues that it does not support the

Appellants arguments because the “assay was done using 293 cells, not peripheral blood leukocytes”.

However, 293 cells are a work horse of modern molecular biology because they are easy to grow and to transfect. ATP and ADP activate TDAG8 in 293 cells to produce cAMP. Given that TDAG8 is naturally expressed in PBLs, there would be no reason to think that activation of the GPCR in PBLs would not lead to an increase in intracellular cAMP accumulation. The Examiner has not presented any evidence to the contrary.

The fact that the assay was performed in 293 cells therefore does not support the Examiner’s position.

The Examiner has mis-read Example 4.

On page 5 of the Office Action, the Examiner states that “It is also noted that Appellants’ argument that increased constitutive activity of TDAG8 leads to an increase in cAMP accumulation is incorrect. Figures 5A and 5B show that increased constitutive activity of TDAG8 leads to an increase in intracellular IP3 accumulation.”

The Examiner’s statement is simply incorrect.

Lines 16-17 of page 41 state that “Exemplary results are presented below in Table I and, in the case of hTDAG8, also in histogram form in Figures 5A (293 cells) and 5B (293T cells).”

Table 1 follows on page 42 and states that hTDAG8 was assayed using the “CRE-LUC assay”. As described in the specification on page 39, the CRE-LUC assay is for determining whether GPCR activation leads to a rise in intracellular *cAMP*. Thus, the results shown in Figs. 5A and 5B show that activation of TDAG8 leads to an increase in intracellular cAMP, not an increase in IP3 as the Examiner argues. These results are also supported by Figs 2A and 2B, which show that ATP and ADP increase cAMP in 293 cells expressing TDAG8, and Figs. 4A and 4B, which show that the amount of cAMP

accumulation is dependent on the dose of ATP and ADP in cells expressing TDAG8.

The Examiner has therefore based this rejection on a mis-reading of the description.

Elevated cAMP accumulation in PBLs inhibits inflammation

At the bottom of page 5 of the Office Action, the Examiner states that Appellants' argument that elevated cAMP accumulation in PBLs inhibits inflammation is unpersuasive, even though the argument is supported by several publications.⁵

According to the Examiner, the Appellants' argument was not persuasive "because Moore et al. teach that cAMP acts as an intracellular second messenger for a variety of hormones, inflammatory mediators, and cytokines." Examples of such compounds include catecholamines, histamines, and prostaglandins.

The Examiner's argument lacks force however, because the fact that many agents can increase cAMP levels in PBLs does not prove or in any way evidence that any individual agent cannot have the same effect. To the contrary, the fact that several agents inhibit inflammatory responses by increasing cAMP levels in PBLs provides more support to the Appellants' argument that ATP and ADP can elevate cAMP in PBLs, and more generally that TDAG8 has a particular biological function of mediating increased cAMP accumulation in peripheral blood leukocytes in response to an agonist.

⁵ See, e.g., Moore et al (Clin. Exp. Immunol. 1995 101:387-389; Naik (Eur. J. Pharmacology 1984 104: 253-259); Deporter et al (Br. J. Pharmac. 1979 65: 163-165); Deporter (Br. J. Pharmac. 1977 60: 205-207); and Bonta (Prostaglandins 1981 22 95-103. References supplied as Exhibits. References are of record.

ATP has a role in inflammation

Finally, on page 6 of the Office Action, the Examiner states that the Appellants' argument that the role of ATP in modulating inflammation is known is unpersuasive, even though the argument is supported by several publications.⁶ Further, Brake et al teaches that screening for a drug for the management of inflammation encompasses screening for an agonist of an ATP-receptor (page 231, right column, lines 24-30).

According to the Examiner, the Appellants' argument was not persuasive "because Brake et al. teach that ATP modulates a plethora of physiological states and cellular responses, including vascular ton, electron transport, mast cell degranulation and synaptic transmission."

The Examiner's argument again lacks force however, because the fact that ATP can have different physiological effects does not prove or in any way evidence that ATP does not have the effect set forth by the Appellants. Further the teaching by Brake et al that an agonist of an ATP-receptor can be useful in the management of inflammation is compatible with the teaching of the instant application that an agonist of TDAG8, by virtue of its elevation of cAMP accumulation, can inhibit inflammation.

The Appellants believe that the facts set forth above support reversal of this rejection, which action is respectfully requested.

⁶ See, e.g., Brake et al (Chemistry and Biology 1996 3: 229-232); Cronstein (J. Appl. Physiol. 1994 76: 5-13); and Daval (Pharmacol. Ther. 1996 71: 325-335). References supplied as Exhibits. References are of record.

II. Claims 69-88 are rejected under 35 U.S.C. §112, first paragraph, because they are rejected under 35 U.S.C. §101. This rejection is applied to new claims 69-88.

This rejection is based on there being no patentable utility for TDAG8. As such, the Appellants respectfully submit that this rejection should be withdrawn along with the §101 rejection for the reasons outlined above.

Reversal of this rejection is requested.

- I. Claims 69-88 satisfy the requirement of 35 U.S.C. § 101.
- II. Claims 69-88 satisfy the requirement of 35 U.S.C. § 112, first paragraph.

The Appellants respectfully request that the rejections of Claims 69-88 under 35 U.S.C. §§ 101 and 112 be reversed, and that the application be remanded to the Examiner with instructions to issue a Notice of Allowance.

Respectfully submitted,

Date: January 18, 2011

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CLAIMS APPENDIX

1-68 (Cancelled)

69. A method of screening for a compound that increases cAMP levels in peripheral blood leukocytes, comprising:

(a) contacting a candidate compound with a G protein-coupled receptor (GPCR) that is present on the surface of a recombinant host cell or isolated membrane thereof, wherein said GPCR comprises an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:82; and

(b) determining that said candidate compound is an agonist of said GPCR; wherein an agonist of said GPCR is capable of increasing cAMP levels in peripheral blood leukocytes.

70. The method of claim 69, wherein said determining step (b) comprises: determining if said candidate compound is a partial agonist of said GPCR.

71. The method of claim 69, wherein said determining step (b) and/or said determining step (c) comprises detecting cAMP.

72. The method of claim 71, wherein said detecting cAMP employs ELISA using an anti-cAMP antibody.

73. The method of claim 71, wherein the recombinant host cell comprises a reporter system comprising multiple cAMP responsive elements operably linked to a reporter gene.

74. The method of claim 71, wherein said detecting cAMP comprises detecting an increase in intracellular cAMP accumulation.

75. The method of claim 69, wherein said determining step (b) comprises using [³⁵S]GTP γ S to monitor G protein coupling to a membrane comprising said GPCR.

76. The method of claim 69, wherein said GPCR comprises an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO:82.

77. The method of claim 69, wherein said GPCR comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO:82.

78. The method of claim 69, wherein said GPCR comprises an amino acid sequence that is at least 99% identical to the amino acid sequence of SEQ ID NO:82.

79. The method of claim 69, wherein the GPCR comprises one or more of the

following amino acid substitutions: P43A, K97N or I130F, relative to SEQ ID NO:82.

80. The method of claim 69, wherein said GPCR is constitutively active.

81. The method of claim 69, wherein the GPCR comprises the following amino acid substitution: I225K, relative to SEQ ID NO:82.

82. The method of claim 69, wherein the method further comprises formulating said agonist as a pharmaceutical.

83. The method of claim 69, wherein the GPCR forms part of a fusion protein with a G protein.

84. The method of claim 69, wherein the host cell is a mammalian host cell.

85. The method of claim 69, wherein the host cell is a yeast host cell.

86. The method of claim 88, wherein the peripheral blood leukocyte is a human peripheral blood leukocyte.

87. The method of claim 69, wherein the recombinant host cell comprises an expression vector which comprises a nucleic acid encoding said GPCR.

88. The method of claim 69, wherein said method further comprises determining whether said agonist increases cAMP levels in a peripheral blood leukocyte.

EVIDENCE APPENDIX

The following references are included in this section. All references are of record.

Benbernou (Immunology 1997 91: 361-368);
Bonta (Prostaglandins 1981 22 95-103);
Brake et al (Chemistry and Biology 1996 3: 229-232);
Chen et al, "Identification of TDAG8 as a P2Y Purinergic Receptor",
abstract of poster;
Cronstein (J. Appl. Physiol. 1994 76: 5-13);
Daval (Pharmacol. Ther. 1996 71: 325-335);
Deporter et al (Br. J. Pharmac. 1979 65: 163-165);
Deporter (Br. J. Pharmac. 1977 60: 205-207);
Eigler (J. Leukocyte Biology 1998 63: 101-107);
Moore et al (Clin. Exp. Immunol. 1995 101:387-389);
Naik (Eur. J. Pharmacology 1984 104: 253-259); and
Neurath et al (Trends Immunol. 2001 22:21-6).

Differential regulation of IFN- γ , IL-10 and inducible nitric oxide synthase in human T cells by cyclic AMP-dependent signal transduction pathway

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SUMMARY

Expression of cytokines by T lymphocytes is a highly balanced process, involving stimulatory and inhibitory intracellular signalling pathways. In the present work, we attempted to clarify the role of cAMP on interferon- γ (IFN- γ), interleukin (IL)-10, IL-4 and IL-13 expression as well as on the inducible nitric oxide synthase (iNOS) expression. Treatment of phytohaemagglutinin (PHA)/phorbol 12-myristate 13-acetate (PMA)-activated Jurkat cells with either dibutyryl-cyclic adenosine monophosphate (cAMP) or pentoxyfylline induced a strong inhibition of IFN- γ mRNA expression as measured by reverse transcription (RT)-polymerase chain reaction (PCR), without affecting IL-10 expression. Both cholera toxin and prostaglandin E₂ (PGE₂) induced a strong inhibition of IFN- γ mRNA expression, whereas IL-10 mRNA expression was significantly enhanced. This differential regulation of IFN- γ and IL-10 expression was related to intracellular cAMP concentration. IL-13 and IL-4 mRNA expressions were not inhibited. We developed a new method based on immunofluorescence for intracellular cytokine detection followed by optical and computerized image processing, and our results showed that IFN- γ protein was strongly inhibited when cells were treated with PGE₂ or dibutyryl (db)-cAMP, whereas IL-10 protein was enhanced. This suggests that cAMP exerts its action at both the transcriptional and protein levels. iNOS mRNA expression was markedly elevated in the presence of PGE₂. The generation of nitric oxide using sodium nitroprusside (SNP) induced a dramatic decrease of IFN- γ , while IL-10 was enhanced; and conversely the inhibition of iNOS activity using L-N^G-monomethyl arginine (L-NMMA) induced a clear inhibition of IL-10 and IL-4, while IFN- γ was enhanced. These results provide evidence that the protein kinase A (PKA) activation pathway plays a prominent role in the balance between the type 1 and type 2 cytokine profile in PHA/PMA-activated Jurkat cells. Data also suggest that iNOS expression is under the control of PKA activation, and that NO seems to be able to assume the polarization of activated T cells to the type 2 profile.

INTRODUCTION

Two types of T helper (Th) cells are distinguished by the pattern of cytokine production. Th1 cell clones produce interleukin (IL)-2 and interferon- γ (IFN- γ), whereas Th2 produce IL-4, IL-5 and IL-10. The cytokine profile determines the effector functions of the two subsets of T cells.¹ Th1 and Th2 cell subsets modulate each other's activity, and the balance between the two subsets determines the outcome of infections

and pathophysiological diseases. Although not mutually exclusive, Th1 and Th2 responses represent alternate functional modes of the immune system. Several recent reports suggest that upon exposure to antigen, CD4⁺ cells *in vivo* do shift their cytokine profile in the context of immune response.² In particular, in response to allergens and parasites, the Th0 cells appears to lose their ability to express IL-2 while enhancing their ability to produce IL-4.^{3,4}

Understanding of the molecular mechanisms that regulate the balance in the expression of Th1 and Th2 type cytokine genes may elucidate fundamental aspects of the immune response.

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Abbreviations: db-cAMP, dibutyryl cAMP; CT, cholera toxin; PTX, pentoxyfylline; L-NMMA, L-N^G-monomethyl arginine; PGE₂, prostaglandin E₂; M-MLV RT, Moloney murine leukaemia virus reverse transcriptase; SNP, sodium nitroprusside; iNOS, inducible nitric oxide synthase.

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3',5'-Cyclic adenosine monophosphate (cAMP) has long been recognized as a second messenger in the control of cellular proliferation.^{5,6} Intracellular cAMP levels may be regulated by both adenylyl cyclase, which synthesizes cAMP,⁷ and cyclic nucleotide phosphodiesterase, which degrades cAMP. cAMP is considered as a potent activator of the protein kinase A (PKA) pathway. It has been demonstrated that cAMP-elevating agents inhibit IL-2 and IFN- γ expression, but

not IL-4 and IL-5 expression.⁸⁻¹⁰ In this regard, we previously reported a differential cytokine profile in peripheral blood mononuclear cells in response to inhibition of phosphodiesterase by pentoxyfilline.¹¹ However, little is known about IL-10 and IL-13 expression.

Nitric oxide (NO) is a short-lived radical that has been identified in recent years as a pleiotropic mediator. It is now admitted that NO plays a critical role in the pathophysiology of several diseases.^{12,13} NO is synthesized from L-arginine by nitric oxide synthases (NOS), which are expressed either as constitutive (cNOS) or inducible (iNOS) enzymes.¹⁴ iNOS is inducible in murine macrophages by proinflammatory cytokines such as tumour necrosis factor- α (TNF- α), IL-1 β , and IFN- γ . Furthermore, iNOS has been shown to be induced by cAMP in rat vascular smooth muscle cells,¹⁵ while controversial results were described in other cell types such as chondrocytes.¹⁶ There is now increasing evidence that NO may be involved in the signalling between macrophages and T cells,¹⁷ although there is still no evidence that the NO pathway operates in human T cells.

For this study, we asked whether iNOS may be considered as a signalling molecule that is differentially implicated in Th1 and Th2 type cytokine expression following stimulation through the PKA signalling pathway. In the current experiments, we examine and compare the effect of cAMP-elevating agents on IFN- γ , IL-4, IL-10 and IL-13 as well as on iNOS expression in Jurkat T cells. Our results further substantiate the fact that intracellular cAMP is an important regulator of cytokine profile expression. We also establish a connection between iNOS expression and the differential sensitivity of Th1 and Th2 cells to cAMP.

MATERIALS AND METHODS

Cell cultures and reagents

The human Jurkat T-cell line was maintained in culture in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 μ g/ml streptomycin, 100 U/ml penicillin (Gibco BRL, Life Technologies, Cergy Pontoise, France). Cells were incubated at 1×10^5 cells/well in 96-well plates, and cultures were stimulated with phytohaemagglutinin (PHA) (10 μ g/ml, Sigma Chemicals, St Quentin Yvelines, France) plus phorbol 12-myristate 13-acetate (PMA) (10 ng/ml, Sigma) for the indicated time points, in the presence or not of prostaglandin E₂ (PGE₂) (Sigma), dibutyryl-cAMP (db-cAMP, Sigma), cholera toxin (CT, RBI, Illkirch, France) or pentoxyfilline (PTX, a generous gift from Hoechst, La défense, France). For certain experiments, cell cultures were performed in the presence of either L-N^G-monomethyl arginine (0.5 mM, L-NMMA, RBI) or sodium nitroprusside (1–10 μ M, SNP, Sigma).

Analysis of mRNA specific for cytokines and iNOS using reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was prepared with TRIZOL reagent (Gibco Life Technologies, France). RNA extraction was performed using phenol/chloroform extraction followed by ethanol precipitation. Precipitated RNA (0.4 μ g) from each sample was reverse-transcribed with oligo-dT as the first-strand cDNA primer and Moloney murine leukaemia virus reverse transcriptase σ M-MLV RT superscript (Gibco Life Technologies)

as previously described.^{18,19} Primer sequences for cytokines and for the internal control β -actin as well as PCR conditions are described elsewhere.¹⁸ Primer sequences for iNOS were as follows: 5'-TCC GAG GCA AAC AGC ACA TTC A-3' for the 5' primer, and 5'-GGG TTG GGG GTG TGG TGA TGT-3' for the 3' primer. Reaction times for PCR were: 94° 1 min, 66.5° 1 min, and 72° 1 min for 35 cycles.

PCR products were denatured and vacuum dot blotted onto Hybond-N⁺ membrane (Amersham, Les Ulis, France). Specific probes were 3'-end labelled with fluorescein-11-dUTP using the enhanced chemiluminescence (ECL) 3'-oligolabelling reagents (RPN 2130, Amersham). Probe sequences for cytokines were described elsewhere,¹⁸ and that for iNOS was as follows: 5'-GGG TTG GGG GTG TGG TGA TGT-3'. Following hybridization to the dot blots and incubation with anti-fluorescein-horseradish peroxidase (HRP) conjugate, the detection of the bound peroxidase was performed using hydrogen peroxide and luminol (RPN 2105, Amersham). The luminescence was detected on blue light-sensitive autoradiography film (Hyperfilm-ECL, RPN 3103, Amersham). The amount of each spot was determined by densitometry analysis (Vilbert Lourmat, Torcy, France). All of the cytokine PCR products were analysed comparatively to the amount of β -actin detected in the same mRNA sample. Separate cycle course experiments confirmed linearity of amplification for β -actin, and cytokine cDNA over 20–35 cycles and 30–45 cycles, respectively. For each PCR, linearity of amplification relative to cDNA dilutions was over 1/5–1/20 for IL-4 and IFN- γ , 1/5–1/40 for IL-13 and 1/5–1/80 for IL-10 and β -actin.

Assay of NO synthesis

After 24 hr of cell incubation, culture supernatants were harvested and the nitrite accumulation was determined by the Griess diazotization reaction (Molecular probe Inc, Eugene, OR,) following a technique previously described.²⁰

Determination of intracellular cAMP concentrations

Cells were harvested at appropriate time incubation, and collected by centrifugation at 400 g. Cells were resuspended in 65% ethanol (v/v). The extracts were centrifuged at 2000 g for 15 min at 4°, and dried in a vacuum oven. A commercially available enzyme immunoassay system (RPN 225, Amersham) was used for experiments.

Immunofluorescence analysis of intracellular cytokines

Cells were incubated for 48 hr in the presence of PHA/PMA, and for the last 8 hr incubated in the presence of Monensin (2 μ M, Sigma) which blocked the intracellular traffic. Optimal intracellular cytokine staining (for both IFN- γ and IL-10) has been achieved using a combination of fixation with paraformaldehyde and permeabilization of cell membranes using Permeafix (OrthoDiagnostics, Roissy, France). Cells were labelled with specific fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (B-T10 for IL-10 and B-B1 for IFN- γ , Diaclone, Besançon, France). Fluorescence was analysed using image processing. Briefly, conventional images of cells were recorded with a Peltier-cooled CCD camera (C5985, Hamamatsu Photonics, Massy, France) through an inverted microscope equipped with epi-fluorescence (Diaphot 300, Nikon, Champigny-sur-Marne, France). Images were digitized using an acquisition video interface in 762 \times 570 pixels

coded in 8 bits, and analysed thereafter in an IBM PC computer using suitable software (Visilog 4, Noesis, Saclay, France). Visilog is a program specializing in image treatment and analysis including image acquisition, filtering and data extraction steps. Fluorescence intensities are mapped in 3D representation using Matlab software (Scientific Software, Sevres, France). Consequently, relative expression of intracellular cytokines in the presence or not of both PGE₂ and db-cAMP was quantified.

RESULTS

Elevating agents of cAMP downregulate IFN- γ but not IL-10 mRNA expression in Jurkat cells

In order to study whether cytokine gene expression could be modulated by the cAMP-dependent signalling pathway, activated Jurkat T cells were incubated in the presence or not of agents that are known to elevate intracellular cAMP. For all the following experiments, Jurkat cells were stimulated with PHA/PMA.

As shown in Fig. 1, a strong inhibition (93%) of IFN- γ mRNA expression, and a significant enhancement of IL-10 (67%) was noticed in stimulated cell cultures incubated for 24 hr with the analogue of cAMP; dibutyryl-cAMP (1 μ M). The same profile of the differential expression of IFN- γ and IL-10 mRNA expression was also observed using cholera toxin (at either the concentrations of 1 or 10 ng/ml).

The involvement of intracellular cAMP in the control of cytokine expression is further confirmed by using pentoxyfilin, which elevates cAMP levels by inhibiting phosphodiesterase and therefore indirectly blocks the degradation of cAMP. As shown in Fig. 1, PTX induced a significant inhibition of IFN- γ mRNA expression relatively to control cells. This effect is dose dependent, and optimal at the concentration of 4×10^{-4} M (54% inhibition, Fig. 1a). Using the same concentrations, no inhibition was observed with the expression of IL-10 mRNA (Fig. 1b).

Using PGE₂ that is known to physiologically elevate intracellular cAMP levels via receptor-mediated activation of adenylyl cyclase enzymes, we showed that IFN- γ was strongly inhibited in the presence of PGE₂ for 24 hr at either the concentrations of 1 and 10 μ M (Fig. 1a), while IL-10 mRNA expression was significantly enhanced (Fig. 1b).

No study has yet investigated the sensitivity of IL-13 expression to elevation of cAMP. Our present data showed that the intensity of the signal specific for IL-13 mRNA was not significantly modified in the presence of db-cAMP at either the concentrations of 1 μ M and 10 μ M (Fig. 1c). A similar profile was obtained with the IL-4 mRNA expression in the presence of the same concentrations of db-cAMP (Fig. 1d).

Regulatory effects of cholera toxin and PTX on cytokine expression are related to elevation of intracellular cAMP

We have investigated whether the differential expression of IL-10 and IFN- γ was in fact due to elevation of intracellular cAMP. Data showed that cholera toxin induced a large increase of intracellular cAMP ($\approx 164\%$ relative to controls). This effect was observed as from 1 hr of incubation period, reached a maximum at 2 hr (Fig. 2), and decreased afterwards

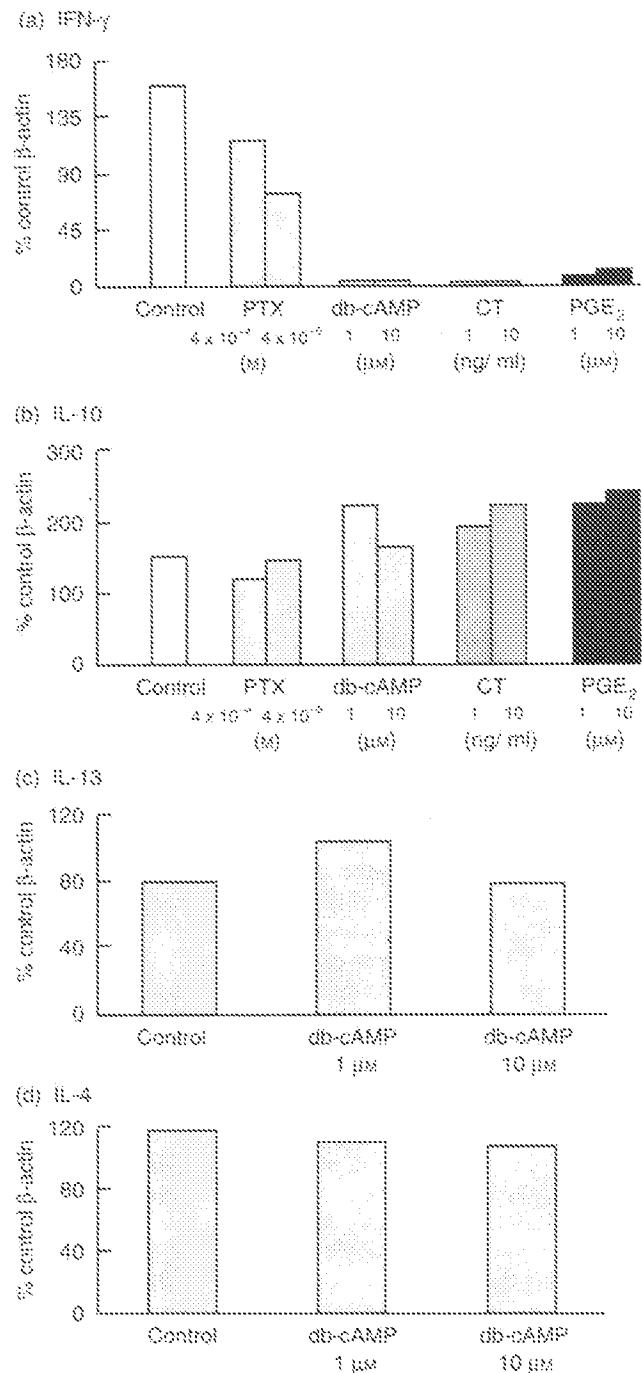


Figure 1. Effect of elevating agents of cAMP on cytokines mRNA expression. mRNA expression for IL-10, IFN- γ , IL-13 and IL-4 was analysed in Jurkat T cells. Cells were incubated in culture with PHA/PMA for 24 hr in the presence or not of db-cAMP, PTX, PGE₂ or CT. The cytokine PCR products were dot-blotted, hybridized with fluorescein-labelled specific probes, then the ECL system detection was used followed by an autoradiography of the chemiluminescence on a sensitive film. The intensity of the signals was determined by densitometry and normalized to β -actin signal. The relative signal intensities obtained from cell cultures of a representative experiment are presented.

(data not shown). A smaller increase (20% relative to controls) was observed with PTX at 4×10^{-4} M after 2 hr of cultures (Fig. 2).

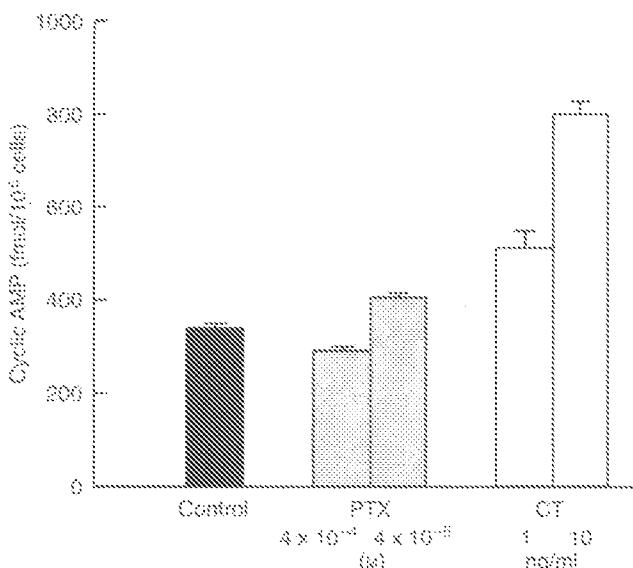


Figure 2. Effect of PTX and cholera toxin on intracellular cAMP. Levels of intracellular cAMP in cultures in the presence of either CT or PTX were determined after 2 hr of incubation. cAMP was measured by ELISA in ethanol extracts. Values represent means \pm SD of three cultures.

Analysis of cytokine expression at the protein level

We next analysed whether the differential expression of IFN- γ and IL-10 observed at the transcriptional level was also obtained at the protein level. A significant increase in IL-10 production after cell treatment with PTX (100 μ g/ml), db-cAMP (1 μ M) or cholera toxin (10 ng/ml) for 24 hr was observed by using enzyme-linked immunosorbent assay (ELISA) (estimated at 2.3-, 3.1- and 3.5-fold relative to control cells, respectively, for PTX, db-cAMP and CT). The measurement of IFN- γ using ELISA did not allow a significant detection even in control cultures (data not shown). In order to improve the cytokine detection, we used a technique based on immunofluorescence for intracellular detection at the single cell level followed by optical and computerized image processing. Jurkat cells were cultured in the presence of PHA/PMA and either db-cAMP (1 μ M) or PGE₂ (1 μ M) for 48 hr. Cell permeabilization and immunolabelling were performed as described in Materials and Methods. Examples of intracellular cytokine detection in Jurkat cells stimulated with PHA/PMA in the presence or not of either db-cAMP or PGE₂ are shown in Fig. 3. Fluorescence intensities specific for IFN- γ protein (Fig. 3a) were highly inhibited when cells were treated with PGE₂ (Fig. 3b) or db-cAMP (Fig. 3c) relative to control cells (Fig. 3a). The magnitude of IFN- γ expression following the effect of either PGE₂ or db-cAMP appeared to be significantly correlated from cell-to-cell analysis. In contrast to IFN- γ , IL-10 production was enhanced in the presence of these agents at the same concentrations (Fig. 3b).

Sensitivity of iNOS expression in Jurkat cells to elevation of intracellular cAMP

When activated *in vitro* with PHA/PMA, Jurkat cells presented significant levels of iNOS mRNA, as measured by RT-PCR.

This expression was markedly elevated by the addition of PGE₂ at the concentrations of either 1 or 10 μ M. Using the same cell preparation, IL-10 mRNA expression was found to be enhanced, whereas IFN- γ mRNA expression was highly inhibited in the presence of PGE₂. Results from a representative experiment are shown in Fig. 4 as autoradiographs. Similar results were obtained when we used the db-cAMP analogue (A two-fold increase of iNOS mRNA expression signal in the presence of db-cAMP relative to control cells, as measured by densitometry). The production of NO in culture supernatants could not be detected using the Griess reagents (data not shown).

These results showed that cAMP-induced elevated iNOS expression associated with the elevation of IL-10 mRNA expression and an inhibition of IFN- γ .

We thus investigated the effect of NO directly on the cytokine profile of Jurkat cells in the conditions of PHA/PMA stimulation. Results showed evidence that the generation of NO using SNP (10 μ M) induced an inhibition of IFN- γ mRNA expression, while the mRNA expression of IL-10 was enhanced (Fig. 5). IL-4 mRNA expression was also enhanced in these experiments (data not shown). Higher doses of SNP (100 μ M) are damaging for cells under our experimental conditions (data not shown).

Finally, we questioned whether the inhibition of NO synthase influences the cytokine expression. Results showed that in the presence of a potent inhibitor of NO synthase (L-NMMA, at 0.5 mM), IFN- γ mRNA expression was enhanced, while IL-10 as well as IL-4 mRNA expression was inhibited (Fig. 6). This further suggests that the activity of iNOS may be important in the control of Th1 and Th2 cytokines.

DISCUSSION

Our study presented here clearly demonstrates that the intracellular cAMP is an important regulator of cytokine profile expression upon mitogenic activation. It was already shown that cAMP inhibits IL-2 and IFN- γ expression but not IL-4 production by T cells in human and rodent systems.^{8,9,11,21-24} More recently, the combination of cAMP elevating agents and PMA was described to upregulate Th2 promoters such as IL-5 in murine cells.¹⁰ However, no study has yet investigated the sensitivity of IL-10 and IL-13 expression to cAMP signalling pathway in T cells. We have used several strategies to stimulate the cAMP signalling pathway, and data indicate that IL-10 mRNA expression in Jurkat T cells was significantly enhanced upon treatment of cells with cAMP analogue, while IFN- γ mRNA expression was clearly inhibited. In the same experimental conditions, IL-13 mRNA expression was not significantly affected as was IL-4 expression. In the presence of CT, a significant inhibition of IFN- γ mRNA expression was found, whereas IL-10 mRNA expression was enhanced as in the case of the cAMP analogue.

A similar profile of the regulation of IFN- γ and IL-10 mRNA expression was also obtained with the more physiological stimulus of PGE₂. These two cytokines are differentially regulated with the same cAMP concentrations. PGEs, which are produced by inflammatory cells such as macrophages,²⁵ are known to activate membrane adenylate cyclase via receptor-gs-protein coupling, resulting in elevation

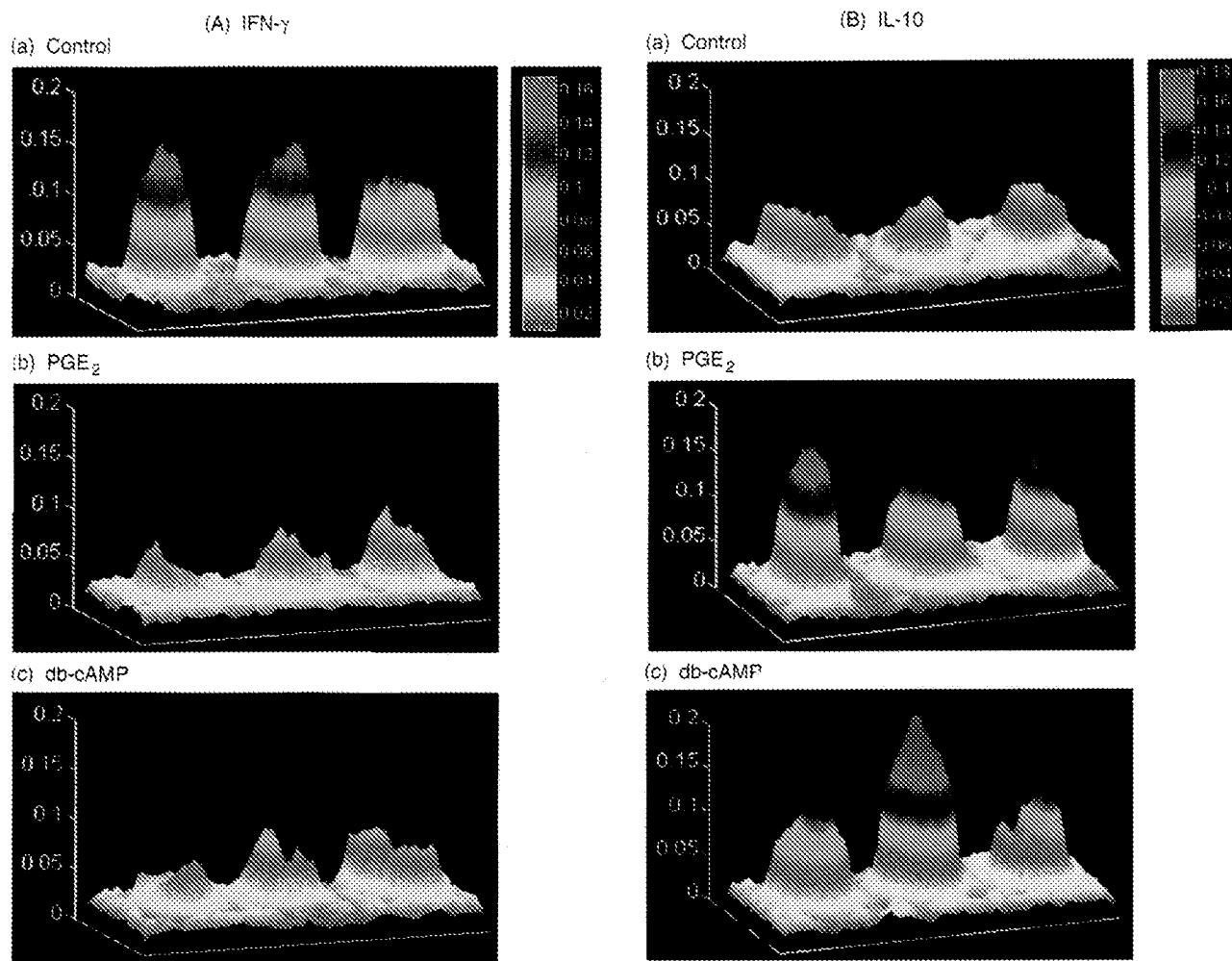


Figure 3. Analysis of intracellular cytokines by immunofluorescence and imaging in cell cultures incubated with PGE₂ and db-cAMP. Intracellular cytokines were analysed in cells stimulated with PHA/PMA for 48 hr in the absence (control) or in the presence of either PGE₂ (1 μ M) or db-cAMP (1 μ M). Cells were permeabilized, and then incubated in the presence of FITC-monoclonal antibody 9mAb) specific for IFN- γ (a) or IL-10 (b). Fluorescence images of the cells were acquired and processed, then pseudocolour 3D mapping of fluorescent cells was performed using Matlab software. Intensities were represented as AU (arbitrary units). These intensities are scaled following a h.s.v. (hue saturation value) colour map. The profile of three different cells was presented. Intensities of fluorescence of cells labelled with the irrelevant mAb (mouse IgG1-FITC, Diaclone) presented values that are always below 0.03 AU.

of intracellular cAMP levels. It has already been shown that elevation of cAMP suppresses some functions including cytokine production and antigen presentation.²⁶ Since the activation of the Th1 cell subpopulation may occur in some inflammatory diseases, one could speculate that the differential sensitivity of Th1 and Th2 cells to PGE₂ may represent at least one regulatory mechanism of inflammatory process.

Cytokine regulation was also analysed at the protein level. Using ELISA, IFN- γ was not detected, while IL-10 production was found to be significantly enhanced upon treatment with db-cAMP, pentoxifylline or cholera toxin. We developed a new image processing system to measure intracellular cytokine expression using specific fluorescent monoclonal antibodies. This method demonstrated a clear inhibition of IFN- γ production in the presence of either db-cAMP or PGE₂, while IL-10 production was enhanced in the same culture conditions. Our results strongly suggest that cAMP exerts its action mainly at the transcriptional level.

The influence of protein kinases on the expression of Th1 and Th2 type cytokine profiles remains to be elucidated. Jurkat cells used in this study produced both Th1- and Th2-type cytokines. Our results demonstrated that under activation of PKA pathway, Jurkat cells are switching to a Th2 profile. However, the extrapolation to how Th1 and Th2 cells themselves may behave following PKA activation remains to be elucidated. Indeed, it is not clear whether our results signify that elevation of IL-10 in response to cAMP is a consequence of IFN- γ inhibition or that cAMP directly triggers IL-10 synthesis at the transcriptional level. Our results are in accordance with the study of Platzer *et al.* indicating that cAMP elevating agents upregulate IL-10 mRNA expression in monocytes.²⁷ In addition, it has already been shown that IL-10 is involved *in vivo* in the protective effect of db-cAMP on endotoxin-induced inflammatory injury.²⁸

In our experimental conditions, we used a combination of PHA and PMA for stimulation. The same profile of the

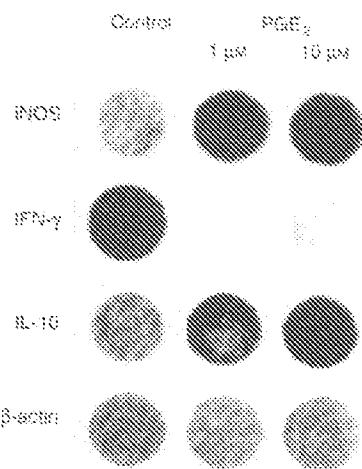


Figure 4. PCR analysis of iNOS mRNA expression in Jurkat cells incubated with PGE₂. Cells were incubated in culture with PHA/PMA in the presence of PGE₂ for 24 hr and mRNA expression of iNOS, IFN- γ and β -actin were analysed. The PCR products were dot blotted, hybridized with a specific probe and detected with the ECL system. The results of a representative experiment are presented as autoradiographs.

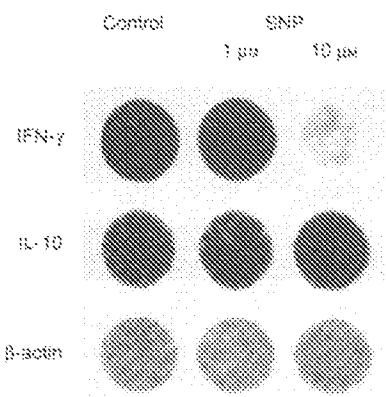


Figure 5. Effect of SNP on IFN- γ and IL-10 mRNA expression. PHA/PMA stimulated cells, in the presence of SNP, were assayed for mRNA expression of IFN- γ , IL-10 and β -actin at 24 hr, as in Fig. 4.

differential control of type 1 and type 2 cytokine expression by cAMP pathway was observed when cells were stimulated with ionomycin and PMA (data not shown). These modes of stimulation mimic T-cell receptor (TCR)-CD3 complex activation, and lead to protein kinase C (PKC) activation and Ca/calcineurin generation that allows NF- κ B activation.

More recently, tyrosine phosphorylation was also described as being a crucial part of the signal transduction pathway in Th1 cells, as these cells presented a TCR-associated protein tyrosine kinases-phospholipase C- γ 1 (PTKs-PLC- γ 1) transduction, while Th2 cells do not utilize this pathway.²⁹

On the basis of our results, we could suggest that elevation of cAMP and consequently, PKA activation could favour a Th2 response in the context of a T-cell response. It is known that allergic patients present an abnormal overexpression of cAMP and PGE₂ levels,³⁰ and increased basal activity of PKA, but decreased basal PKC activity.³¹ Furthermore, PGE₂ promotes B lymphocyte immunoglobulin isotype switching to

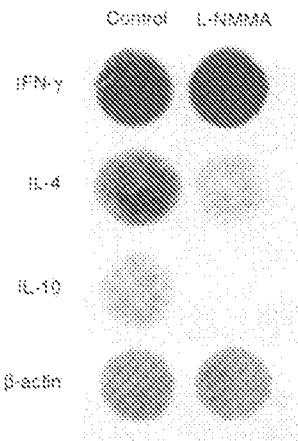


Figure 6. Effect of L-NMMA on cytokine mRNA expression. PHA/PMA stimulated cells, in the presence of L-NMMA, were assayed for mRNA expression of IFN- γ , IL-4, IL-10 and β -actin at 24 hr, as in Fig. 4.

immunoglobulin E (IgE).³² So, it seems possible that macrophage production of PGE₂ may regulate both T and B cells, stimulating differentiation of Th2 cells as well as promoting IgE production.

NO is an important mediator in the control of physiological processes, and seems to be implicated in the pathogenesis of several diseases.¹⁴ NO is largely produced by cytokine-activated mouse macrophages, fibroblasts, chondrocytes and endothelial cells,³³ although, there is still no evidence that NO pathway operates in human T cells. Our data showed for the first time that iNOS expression occurs in Jurkat T cells, and is influenced by elevation of intracellular cAMP. Indeed, iNOS mRNA expression was clearly enhanced in the presence of PGE₂ (Fig. 4). These results are in accordance with other recent reports indicating the induction of iNOS via elevation of cAMP concentration in different types of smooth muscle cells¹⁵ and rat mesangial cells.³⁴ However, controversial results were recently described in human articular chondrocytes.¹⁶

The differential expression of iNOS in Jurkat cells may be due to a direct effect of cAMP or it may be a feature of Th2 cells. However, this may be somewhat inconsistent with a previous report of Taylor Robinson indicating that murine Th1 cells, but not Th2 cells, can be activated by antigens or mitogens to produce large amounts of NO.³⁵ Furthermore, Thüring *et al.* state that iNOS is not present in T-cell clones and T lymphocytes from naive and *Leishmania major*-infected mice.³⁶ However, in this latter paper, iNOS expression was investigated as an enzymatic activity and at the protein level, and was not analysed by RT-PCR. On the other hand, we could not detect NO production in culture supernatants by using the Griess colorimetric assay (data not shown). So, we suggest that this latter technique may be not sufficiently sensitive to allow detection of a weak NO production as in the case of our experimental conditions.

It may be possible that the increase of iNOS contributes to the inhibition of IFN- γ expression in addition to an autocrine activity of the Th2 cytokines such as IL-10, and perhaps, IL-4. This hypothesis is supported by the fact that the generation of NO using SNP in our experimental conditions induced an increase in IL-10 production, which is

associated with a dramatic decrease in IFN- γ mRNA expression (Fig. 5). In addition, when we used l-NMMA to inhibit iNOS, the IL-10 expression as well as IL-4 expression was almost completely inhibited, while IFN- γ was highly expressed. This is in accordance with the study of Taylor-Robinson *et al.* in murine clones indicating that exogenous NO inhibits IFN- γ production.³⁵ These latter data further substantiate the fact that iNOS expression is not only closely implicated in Th2-type response, but also antagonizes the type 1 response.

It is known that IFN- γ acts as a potent co-stimulus for iNOS expression, and that it completely inhibits the IL-10 response.³⁷ Furthermore, it has already been shown that IL-10 and IL-4 inhibit iNOS in response to IFN- γ in macrophages.³⁸ This signifies that iNOS expression levels may be balanced by positive and negative influences during interaction between macrophages and T cells.

There is evidence that nuclear factors are involved in the differential regulation of cytokine genes. A recent study from Arai and co-workers on the murine cell line EL-4 indicated that NF-AT-related complexes are involved in the differential regulation of IL-2 and IL-5 genes by cAMP.³⁹ Another study reported the implication of the competition between RelA and NF-ATp on the inhibitory effect of PKC on IL-4 but not IL-2 expression in Jurkat cells.⁴⁰ It was also shown that Th2 cells, in contrast to Th1 cells, are lacking in nuclear translocation of NF- κ B.⁴¹ NF- κ B was also described as being closely related to iNOS expression,⁴² and that NO was found to be able to mediate the regulation of the AP-1 transcriptional factor.⁴³ The molecular mechanisms of cAMP action on the differential expression of cytokine genes in our experimental conditions and the sequential implication of NO remained to be elucidated.

Taken together, our results provide evidence that PKA activation pathway plays a prominent role in the balance between type 1 and type 2 cytokine profile in activated Jurkat cells. We also show that iNOS mRNA expression is under the control of PKA activation and is associated with the Th2-type profile, and that NO is also able to polarize activated T cells to Th2-type profile.

ACKNOWLEDGMENTS

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PROSTAGLANDINS

PROSTAGLANDIN E₂ ELEVATION OF CYCLIC-AMP IN GRANULOMA MACROPHAGES AT VARIOUS STAGES OF INFLAMMATION: RELEVANCE TO ANTI-INFLAMMATORY AND IMMUNOMODULATORY FUNCTIONS.

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Abstract

In the carrageenin-induced granuloma of rats the inflammatory tissue growth and macrophage invasion on the one hand and the cyclic-AMP content of the macrophages on the other, display opposite directional changes. Macrophages, isolated from this tissue at different stages of inflammation, were used to examine the effect of prostaglandin E₂ on intracellular levels of c-AMP. It appears that during infiltration of the macrophages into the inflammatory tissue, the sensitivity of adenylate cyclase to activation by PGE₂ increases. Arguments are presented that these observations made *in vitro*, are in direct relevance to the previously described anti-inflammatory effect of PGE on granuloma tissue *in vivo*.

Introduction

Prostaglandin E inhibits several functions of cultured macrophages of bone marrow or peritoneal origin (1,2). On the basis of indirect evidence, including the knowledge that PGE is an activator of adenylate cyclase (3), it has been suggested that this inhibition of certain macrophage functions is partially mediated by a rapid increase in the intracellular level of c-AMP (4). However, in these papers little attempt was made to associate the observations on cultured cells with the anti-inflammatory effect of PGE *in vivo*. In this respect, experimental granuloma, in which infiltrating macrophages are major cell constituents and which is a characteristic model of the chronic, i.e. tissue-proliferative component of inflammation, is reduced by PGE₁ and PGE₂ (5,6).

We now report the PGE₂-induced elevation of c-AMP in macrophages derived from granuloma, at a time period which is critical for the appearance of the *in-vivo* anti-inflammatory effects of E-type prostaglandins. We propose that during the development of granuloma the sensitivity of macrophages to PGE₂-induced elevation of c-AMP increases and that this change is relevant to the anti-inflammatory effect of PGE on granuloma. Some preliminary results of this work were presented at a workshop (7).

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PROSTAGLANDINS

Methods

Induction and quantitation of granulomatous inflammation by subcutaneously implanted carrageenin-soaked sponges in rats were carried out as described earlier (8). Cells were obtained as follows: On different days of inflammation rats were killed by crushing the spinal cord at the height of the neck, the sponges plus granuloma were removed, the tissues placed in Gey's Balanced Salt Solution (GBSS) on ice and cut into pieces. The extracellular tissue matrix was removed by digestion for 1h at 37°C with Pronase E (Merck, Darmstadt; 70.000 PUK/gr). After filtration, centrifugation for 5 min at 300xg, resuspension in GBSS and hypo-osmolarity lysis of erythrocytes, the remaining cells were washed three times, the total number of viable cells (trypan blue exclusion) were counted and a dilution of 5×10^6 cells/ml GBSS was made for incubation. Smears for differential cell counts were stained with May-Grunwald-Giemsa solution.

One ml samples of 5×10^6 cells were incubated with or without PGE₂, for 15 min at 37°C. Thereafter, EDTA (4 μ M final concentration) was added at 4°C and 100 μ l samples were taken for protein assay by the method of Lowry et al. (9). The cells were sonicated and after 5 min in a boiling waterbath, the particulate material was removed by centrifugation at 300xg for 5 min. The supernatant was stored at -70°C for subsequent c-AMP assay. To this end, the samples were resuspended in 400 μ l Tris/EDTA buffer (pH 7.5) and 100 μ l aliquots were taken to measure c-AMP by the protein binding method (10), using a commercial kit (Radiochemical Centre, Amersham). Several cell suspensions, particularly those which were obtained from tissues at an early stage of development, contained a mixed population of macrophages and granulocytes. Since however, the macrophage/granulocyte ratio was known from the differential cell count, the measured values of c-AMP were corrected for the proportional number of macrophages and expressed as a content in 10^6 macrophages. In order to verify these results, in some experiments, prior to c-AMP assay, the cell suspension was subjected to density-gradient centrifugation on Ficoll-Isopaque (density 1.077 g/ml, Nyegaard, Oslo). From the two cell layers thus obtained, the upper layer contained a 90 percent macrophage population. In all experiments, reported in this paper, theophylline (0.5 mM) was added to the incubation medium, in order to prevent any possible deterioration of c-AMP. Preliminary experiments, under such conditions, have shown that irrespective whether 10 or 15 minutes incubation with PGE₂ was carried out, the elevation of c-AMP was identical. In the experiments, shown in this paper, the c-AMP was measured following 15 minutes incubation of the cells with PGE₂.

Results

During the development of granulomatous inflammation the number of macrophages in the tissue progressively increased (at the expense of granulocytes, data not shown) and in fact, the proportional increase in the macrophage count paralleled the tissue growth in terms of

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granuloma dry weight. The peak value for both parameters was observed 6 days after induction of inflammation. The c-AMP content of macrophages was at its highest in the cells isolated during the early phase of inflammation and was progressively lower in cells derived from granuloma tissue during its later development. The inverse relation between the c-AMP levels in macrophages and the growth of inflammatory tissue is shown in Fig.1.

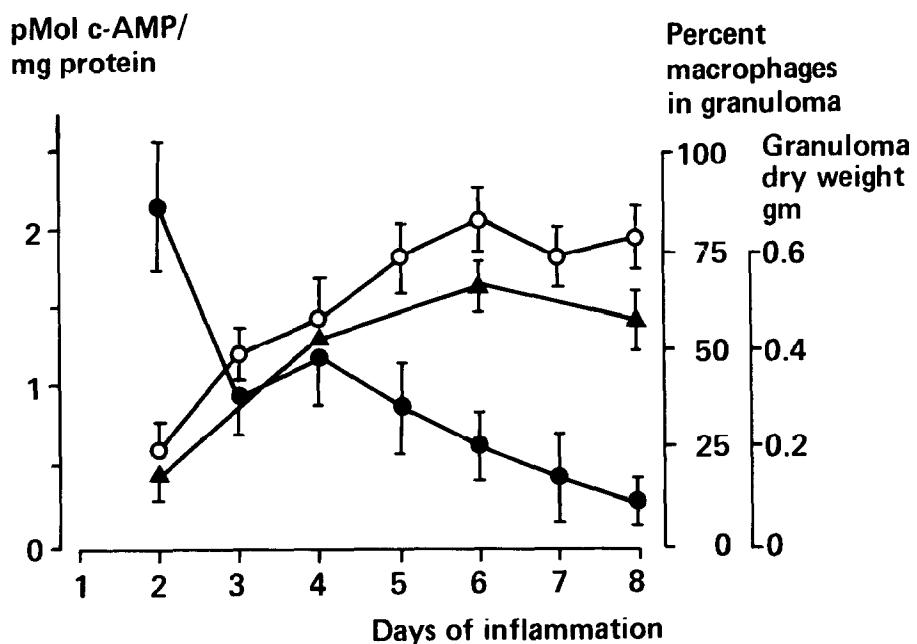


Fig. 1. Granuloma dry weight (▲), percent macrophages (○) and c-AMP content of macrophages (●). Each point represents the mean of three experiments (two rats each) + S.E.M. Theophylline (0.5 mM) was added to the incubation medium.

These c-AMP levels were calculated on the basis of the proportional number of macrophages in the tissue. That the downward trend with time was a function of changes in the macrophages and not in other contaminating cells was verified by measurements on 90 percent macrophage populations, obtained by density-gradient centrifugation of cells from 3- and 6-day granulomata. Thus in macrophages obtained from 3-day inflammatory tissue the c-AMP content was 5.28 pMol/mg protein vs 3.72 pMol/mg protein in macrophages from 6-day tissue.

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We have found previously that the anti-inflammatory, i.e. granuloma-reducing, effect of PGE is restricted to administration at a well-advanced stage of granuloma growth (5,6). Furthermore, the use of an antagonist suggested that this effect might have been mediated through activation of adenylate cyclase (11). Therefore we examined the influence of PGE₂ on c-AMP levels of macrophages isolated at different days of granuloma growth. In agreement with the stimulating effect of E-prostaglandin on adenylate-cyclase, there was a marked increase in c-AMP following 15 minutes exposure of the cells to PGE₂. However, the magnitude of the increase was dependent on the stage of development reached by the granuloma tissue from which the cells were removed. Thus, PGE₂ at all doses achieved optimal stimulation of c-AMP content in macrophages which were isolated from 4 day granuloma tissue (Fig.2).

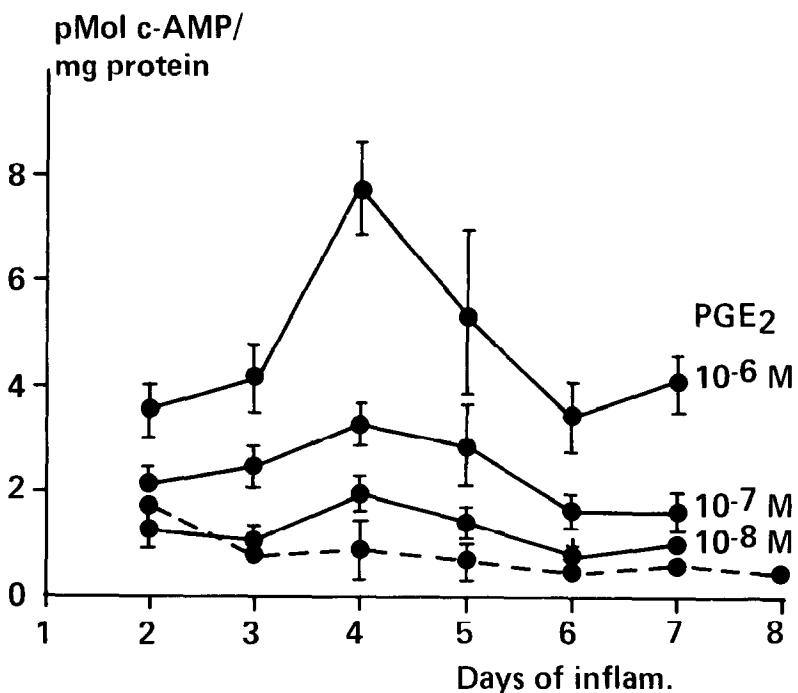


Fig. 2. Effect of PGE₂ on the c-AMP content of macrophages isolated from granuloma tissue on different days of inflammation. The cells were exposed for 15 min to PGE₂ at the concentrations indicated. The dotted line indicates the control cell suspension to which saline was added. The experiments were carried out in the presence of theophylline (0.5 mM). Each point represents the mean + S.E.M. of duplicate measurements on four cell suspensions each of which were obtained from four rats. Significance was calculated by one-tailed Mann Whitney U-test. For PGE₂ 10⁻⁶M, at each point: P<0.02; for 10⁻⁷M on days 3,4 and 5: P<0.002, days 6 and 7: P<0.05; for 10⁻⁸M on day 5: P<0.05. Other points P<0.05.

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When expressing the effects in terms of percent change from saline-treated macrophages, the dose-response curves thus obtained clearly show that the sensitivity of macrophages to PGE₂-induced elevation of c-AMP content was greater when the cells originated from well-advanced, i.e. 4 to 7 day granulomata than when they were isolated from poorly-developed, i.e. 2 to 3 day tissues (Fig. 3).

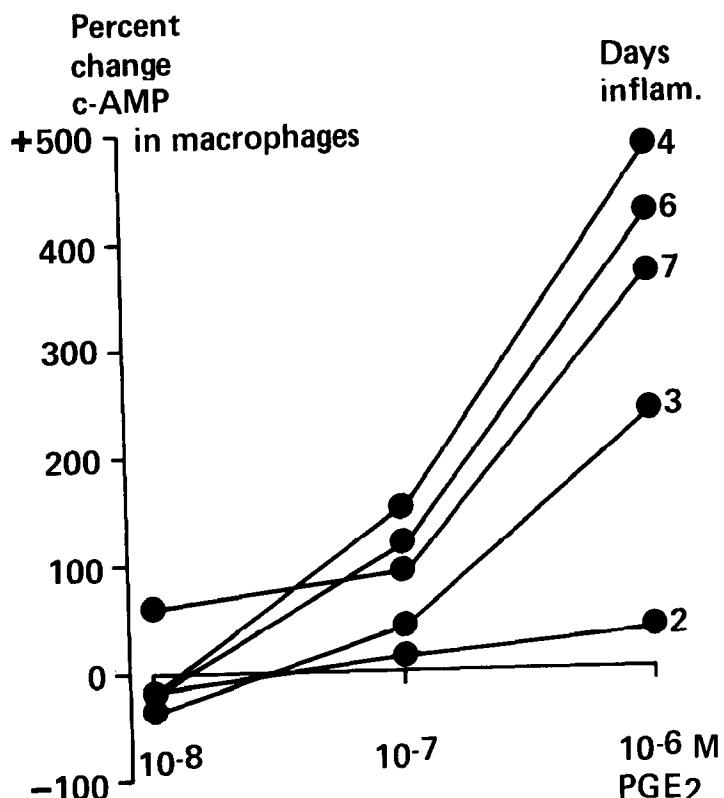


Fig. 3. Dose-response curves of effects of PGE₂ on c-AMP content of macrophages. These curves are derived from the experiments shown in Fig. 2. Each point represents the response to a single dose of PGE₂ on macrophages removed on the day of inflammation shown, expressed as a percentage of the c-AMP concentration in the same cells incubated with saline.

In a separate experiment in macrophages isolated from 4 day granuloma tissue the effect of PGE₂ was examined in the presence of 9-tetrahydro-2-furyl-adenine (SQ 22536), which is an inhibitor of adenylate cyclase (12). The results, as shown in Table I, indicate that the inhibitor of adenylate cyclase markedly counteracted the PGE₂-induced rise in c-AMP levels.

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Table I. Effects of PGE₂ and SQ 22536 on c-AMP levels of macrophages from 4-day granuloma.

Substance and concentration ^{a)}	c-AMP percent change ^{b)}
PGE ₂ (3 x 10 ⁻⁶ M)	506 ± 46 increase
SQ 22536 (10 ⁻³ M)	42 ± 12 decrease
PGE ₂ + SQ 22536	143 ± 73 increase ^{c)}

a) 15 min incubation.

b) Values represent the mean ± S.E.M. as compared to c-AMP level in the same cell suspension incubated with saline. Duplicate measurements were made on four cell suspensions which were pooled from 2 rats.

c) Significance vs PGE₂ alone P<0.025 (Mann Whitney U-test).

Discussion

Macrophages play an integral role in chronic inflammatory processes (13). E-type prostaglandins inhibit the locomotion, shape change, phagocytosis by, chemiluminescence from and colony formation of cultured macrophages of peritoneal cavity or bone marrow origin (1,2). Bone marrow or peritoneal elicited macrophages exposed to PGE₁ or E₂ respond with elevated levels of c-AMP (4,14,15). Both PGE₁ and E₂ also counteract chronic inflammation in a variety of experimental models *in vivo* (16). However, none of the authors who demonstrated these actions of PGEs on macrophages *in vitro*, made any efforts to relate these effects to the inhibiting influence of E-prostaglandins on chronic inflammation *in vivo*. We consider that the conditions and results of the *in-vitro* experiments reported here are such as to justify their bearing on the anti-inflammatory effect of PGE in the granulomatous model of chronic inflammation. The arguments in favour of this correlation are as follows: Firstly, the macrophages in these experiments were isolated from the same granuloma tissue as that on which the anti-inflammatory effects of PGE₁ and E₂ have previously been demonstrated (5,6). Secondly, because in this particular model, the inflammatory tissue growth and macrophage-invasion on the one hand and the c-AMP content of the macrophages on the other, display opposite directional changes. This finding is strongly suggestive of the existence of a functional interrelationship. Thirdly, taking into account the time-scale of this inflammatory model, the change in sensitivity of the macrophages to PGE₂-induced elevation of c-AMP, strikingly resembles the change in sensitivity of granuloma tissue to the anti-inflammatory effect of PGE. More explicitly, in the present experiments the effect

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of PGE₂ on the c-AMP level was considerably more pronounced with macrophages isolated from 4 to 7 day granulomata than with those originating from the tissue prior to day 4 (see Fig.3). Similarly, days 4 to 7 proved the critical period during which local administration resulted in a granuloma-reducing, antiinflammatory effect with either PGE₂ (6) or PGE₁ (5). The parallelism is sufficiently strong to permit the proposal that the presently observed *in-vitro* and the earlier reported *in-vivo* responses are interrelated. Fourthly, the relevance of the PGE₂-induced rise in the c-AMP of macrophages *in vitro* to its anti-inflammatory effect *in vivo* is also supported by the earlier observation, showing that the granuloma reducing effect of PGE₁ was abolished by the concomitant administration of the substance SQ 22536, an inhibitor of adenylate cyclase (11).

The fall in the c-AMP content of granuloma-derived macrophages was initially detectable at a period during which the infiltration of the macrophages was still in progress (vide Fig.1). Furthermore, the largest increase in c-AMP level following exposure to PGE₂ occurred in macrophages which were isolated at a period when the infiltration of these cells into untreated granuloma tissue would still have been progressing. The reason of this requires clarification, but it is worth noting that macrophage derived PGE₂ stimulates c-AMP accumulation in the same cells from which it is produced and that macrophages from 7-day carrageenan granuloma form much less PGE₂ than normal cells (17, 18). Perhaps this fall in endogenous PGE₂ production by granuloma macrophages may partially help to explain both the gradual fall in granuloma macrophage c-AMP levels and the increasing sensitivity of these cells to exogenous PGE₂.

Macrophages not only play an integral role in chronic inflammation (13), but are also a non-specific branch of the immune system (19). Carrageenin has been adopted as a tool for studying the role of macrophages in the expression of immune reactivity (20). Macrophages from carrageenin-induced granulomas do have properties, e.g. proneness to bind soluble IgG aggregates and capacity to secrete a thymocyte stimulating factor (21), which render such macrophages similar to those which are activated by immune mechanisms. Prostaglandins are modulators of chronic inflammation (16) and they have been proposed as regulators of immune responses (22). Furthermore, a close link between these two functions of prostaglandins has been suggested (16). Because with granuloma-derived macrophages the *ex-vivo* responsiveness of macrophages to PGE appears to be relevant to the responsiveness of the carrageenin-granuloma to PGE *in vivo*, such combined studies may be of considerable benefit for the further investigation of a link between the anti-inflammatory effects and the putative immune-regulatory functions of prostaglandins.

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Acknowledgements

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ATP receptors in sickness, pain and death

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Extracellular ATP elicits biological responses ranging from cell death to synaptic transmission. Recent gene-cloning efforts have uncovered a family of cell-surface ATP receptors, which are potential targets for the development of novel drugs to treat airway and cardiovascular diseases, inflammation and pain.

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Adenosine triphosphate (ATP) is best known as the molecular currency of intracellular energy stores. Less well appreciated is its multifaceted role as an extracellular signaling molecule acting through cell-surface receptors. Within the cardiovascular, immune, and nervous systems, ATP modulates a plethora of physiological states and cellular responses, including vascular tone, electrolyte transport, mast cell degranulation, and synaptic transmission in the central nervous system and periphery. Physiological and pharmacological studies have convincingly shown that ATP exerts its actions by binding to a family of functionally distinct cell-surface receptors, known as purinergic type 2, or P₂, receptors [1]. This is a notable achievement given that there are almost no receptor-subtype-selective antagonists or radioligands available to aid in the characterization of ATP-mediated responses or ATP-binding sites. Despite the limitations imposed by this lack of pharmacological tools, it was proposed several years ago [2,3] that ATP resembles certain other neurotransmitters in its ability to activate both G-protein-coupled receptors (the P_{2U}, P_{2Y}, and P_{2T} subtypes) and ligand-gated ion channels (P_{2X} and P_{2Z} subtypes), and that these receptor types could be distinguished by their agonist sensitivities and signal-transduction mechanisms. These predictions have been borne out by the recent characterization of functional cDNA clones encoding numerous members of the ATP-receptor family.

Molecular characterization of ATP receptors

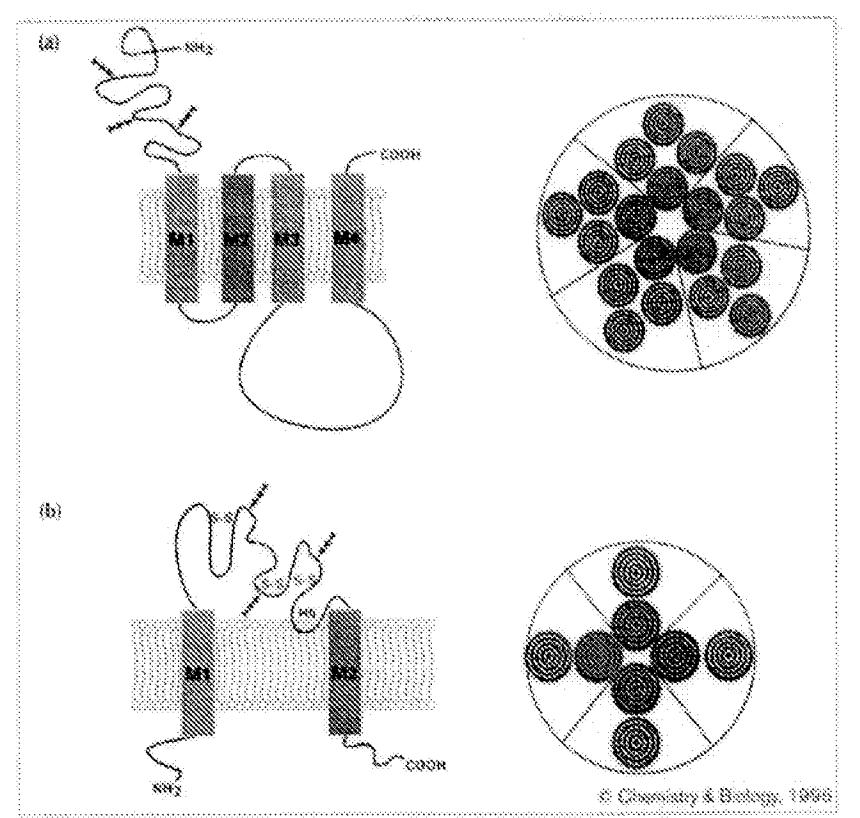
The first members of the P₂-receptor family to be cloned corresponded to the P_{2U} and P_{2Y} subtypes, as evidenced by their pharmacological properties when expressed in *Xenopus* oocytes [4,5]. Examination of their deduced protein sequences showed them to be members of the superfamily of G-protein-coupled receptors, forming a distinct branch of the family that is more closely related to receptors for platelet-activating factor, angiotensin II, interleukin-8A and thrombin than to receptors for adenosine

and cAMP. Other members of this gene family have now been cloned from a variety of tissues and organisms. Functional analysis of these cloned receptors reveals a diversity of subtypes beyond that previously detected using available pharmacological tools. Although these cloned receptor subtypes all share the ability to transduce signals via the activation of phospholipase C and the mobilization of intracellular calcium, they are differentially sensitive to a variety of nucleotide agonists. To accommodate this new molecular information, a revised nomenclature has been proposed [6] for purinergic receptors, in which G-protein-coupled purinergic receptors are designated P_{2Y1}, P_{2Y2}, etc., as a function of their sequence similarity (an updated compendium of purinergic receptors is currently maintained at a site on the World Wide Web (<http://mgddk1.niddk.nih.gov:8000/>)).

Perhaps the most surprising outcome to arise from the molecular cloning of ATP receptors has been the recent discovery of a family of P_{2X} receptors that defines a novel structural motif for ligand-gated ion channels. Many excitatory and inhibitory ligand-gated ion channels share a common subunit structure in which a large amino-terminal, extracellular, ligand-binding domain is followed by four transmembrane segments. For the best characterized member of this channel superfamily, the nicotinic acetylcholine receptor (nAChR), five subunits assemble to delineate a central water-filled ion pore. Because the biophysical properties of native P_{2X} receptors resemble those of nAChRs, it was expected that these proteins would have a common structural design. But the deduced protein sequences of the first two ATP-gated channel subtypes to be cloned (P_{2X1} from rat vas deferens smooth muscle and P_{2X2} from the PC-12 rat pheochromocytoma cell line) suggest a subunit and receptor structure that is quite different from that of the nAChR [7,8] (Fig. 1). Each of these P_{2X} subunits is predicted to have a topology in which two transmembrane segments are separated by a large extracellular ligand-binding loop, with amino and carboxyl termini residing within the cytoplasm.

Although the P_{2X} receptors bear no sequence similarity to other signal transduction proteins, their predicted topological organization is reminiscent of that proposed for the family of voltage-insensitive ion channels [9], which include inwardly-rectifying and pH-sensitive potassium channels, amiloride-sensitive sodium channels and presumptive mechanosensory ion channels from worms. Recent studies suggest that these channel complexes may be composed of four subunits, but the stoichiometry of functional ATP-gated channels has yet to be determined.

Figure 1



Predicted structures of (a) a nAChR and (b) an ionotropic ATP receptor. In both cases the M2 transmembrane segment is predicted to form the ion-conducting pore. The subunit stoichiometry of the nAChR is well established, while that of the ionotropic ATP receptor remains speculative. Arrows represent glycosylation sites.

When the first two P_{2X} receptors were cloned, the only entry in the sequence databases showing any sequence similarity was a partial cDNA clone called RP-2; this clone was isolated in a screen designed to identify genes whose expression is induced during programmed cell death in rat thymocytes [10]. As it turns out, RP-2 encodes a protein corresponding to $P_{2X}1$. In the light of previous observations that extracellular ATP can elicit programmed cell death in thymocytes and other cell types (e.g., see [11]), this cloning convergence provides additional evidence to support the intriguing notion that ATP might act as a paracrine or autocrine signaling molecule during apoptotic death in some physiological systems.

Cloned cDNAs representing at least six different P_{2X} subtypes have now been reported (see [12] and references therein) and all are capable of forming functional homomeric channel complexes when expressed in *Xenopus* oocytes or transfected mammalian cells. In general, these channels resemble native P_{2X} receptors in showing a lack of selectivity among small monovalent cations, a reversal potential near zero millivolts, and an inward rectification of their current–voltage relationships. Some channels also exhibit a relatively high permeability to calcium ions and a marked potentiation of ATP-elicited current responses in

the presence of extracellular zinc. These properties may be important for the function of P_{2X} receptors in the long-term modulation of cellular processes such as cell death (apoptosis) or synaptic plasticity.

Properties of these heterologously expressed cDNAs appear to account for many, but by no means all, of the diversity of ATP-gated channels studied in various tissue preparations. This suggests that there are still more subtypes to be cloned and/or that some of the native receptors are heteromeric complexes formed by association of different subunits. The formation of heteromers between $P_{2X}2$ and $P_{2X}3$ receptors has been observed; co-expression of these two subunits results in the formation of ATP-gated channels with properties different from those of either of the homomeric channels [13,14]. The properties of these heteromeric channels resemble those reported for channels found in sensory neurons of the nodose ganglion and dorsal root ganglion (namely a slowly desensitizing response to α,β -methylene ATP) [15]. As discussed below, the sensory nervous system represents a particularly interesting opportunity for molecular biology to clarify the subunit composition of native ATP-gated channel complexes within the pain signaling pathway. Defining the structure and expression of P_{2X} receptors will provide a

basis for the rational design of novel therapeutic agents for the control of pain.

ATP and pain

It has been over forty years since Holton and Holton [16] suggested that ATP released from sensory neurons is important in synaptic transmission. Building on this early observation, other investigators sought to establish that ATP acts as a neurotransmitter, directly activating the dorsal horn neurons that are responsible for pain transduction in the spinal cord. Early studies were limited by the fact that ATP is actively metabolized to adenosine in the extracellular space. Adenosine is now recognized as an inhibitory transmitter in the dorsal horn of the spinal cord, acting through its own family of purinergic (P_1) receptors [17,18]. With the development of neuronal cell culture preparations and patch-clamp recording methods, ATP has been unambiguously identified as a direct activator of ion channels on spinal cord dorsal horn neurons and primary afferent neurons from a variety of sources, including both somatic and visceral sensory ganglia [19].

What might be the role of these channels in the sensory transduction of pain, both at the site of tissue damage and at spinal cord synapses that relay sensory information to the brain? ATP released from damaged cells into the extracellular space could activate primary afferent nociceptors, specialized sensory neurons that transduce intense thermal, mechanical, and noxious chemical stimuli into the electrochemical signals of pain. This hypothesis is supported by the observation that ATP induces pain in a human blister-based model [20]. Direct evidence for the selective expression of ATP-gated channels in sensory neurons has been provided by the cloning of several P_{2X} subtypes. As mentioned above, the P_{2X3} subtype, whose expression is restricted to sensory neurons [14], can assemble with the more widely expressed P_{2X2} subunit. Together, these subtypes form ATP-gated channels whose properties resemble native receptors found on sensory neurons [13]. As these cloned receptor subunits have just become available for study, their role in signal transduction in nociceptors and other sensory neurons can now be more fully explored.

The notion that ATP has a role in the spinal cord pain pathway is supported by observations that the P_2 antagonist suramin can reduce pain transduction in rats when placed in the cerebrospinal fluid at the level of the spinal cord. Similar administration of the P_{2X} receptor agonists α,β -methylene ATP or 2-methylthio ATP reduces the latency of a pain response to a heat stimulus applied to the tail, which is interpreted as enhancing pain signal transduction [21]. At the molecular level, most P_{2X} receptor subtypes cloned to date show significant expression within the spinal cord [12], including superficial layers of the dorsal horn, where primary afferent nociceptors make

synaptic connections with spinal interneurons. These interneurons connect with both local reflex loops and ascending pathways that transmit sensory signals to the brain, where they are ultimately perceived as pain. Whole-cell electrophysiological recordings from a subset of dorsal horn neurons suggest that ATP is an enhancer of the excitatory actions of the neurotransmitter glutamate, but it remains possible that ATP is itself a principal excitatory agent within the spinal cord [22].

A new frontier for drug design

ATP is fast being recognized as an important extracellular signaling molecule in a vast array of biological processes. Nevertheless, the pharmacopœia available for the study and manipulation of ATP receptors is still rather primitive. As molecular studies show, the ATP-receptor family is more complex than previously appreciated, and the need for receptor-subtype-selective drugs has become all the more apparent. For example, the P_{2X4} receptor subtype is relatively insensitive to many compounds commonly used to define P_{2X} sites [23]. Thus, identifying fast, excitatory responses to ATP *in vivo* will require the development of new compounds that can distinguish synaptic events mediated by ATP from those elicited by other excitatory transmitters. Further motivation for the development of specific and potent ATP-receptor agonists and antagonists is provided by the possibility that these drugs may be useful in the management of common physiologic disorder, including pulmonary and cardiovascular disease, urologic dysfunction, inflammation and pain syndromes.

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Identification of TDAG8 as a P2Y Purinergic Receptor

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SEQUENCE ALIGNMENT

human P2Y1	SILPFCTISAHRY
human P2Y2	SILPFCTISVHRC
chicken P2Y3	SILPFCTISVQRY
human P2Y4	SIVLFCTISVHRY
human P2Y5	SILPFCTISVDRF
human P2Y6	SILPFCTISVQRY
xenopus P2Y8	SILPFCTISVDRF
human P2Y9	SICPFCTISVQRC
human P2Y10	SIVPFCTISVNRY
human P2Y11	SITPLFCGAVDRY
human TDAG8	

Fig. 1 Sequence alignment of P2Y receptors and TDAG8. Amino acids at the carboxyl-terminal of transmembrane domain 3 are shown. Conserved amino acids are in bold and underlined.

INTRODUCTION

T-cell-associated gene 8 (TDAG8) was originally identified by its increased expression during activation-induced cell death (ACID) of thymocytes. TDAG8 encodes a putative G protein-coupled receptor whose ligand is unknown. We have demonstrated that TDAG8 is a receptor for the nucleotides ATP, ADP and UTP. HEK293 cells transfected with a human TDAG8 expression vector have elevated levels of cAMP, suggesting that TDAG8 is coupled to Gs and is constitutively active. Addition of ATP and ADP causes a further increase of intracellular cAMP in a dose dependent manner, indicating that TDAG8 is a purinergic receptor. As a newly identified member of the P2Y purinergic receptor subfamily, TDAG8 is unique in that it is preferentially coupled to the adenylyl cyclase pathway and does not activate inositol phosphate production in response to nucleotides. The restricted expression of TDAG8 in lymphoid tissues, its induction during ACID of thymocytes, together with the fact that extracellular ATP can cause apoptosis of thymocytes, suggest that TDAG8 may play a role in mediating thymocyte apoptosis.

LIGAND POTENCY

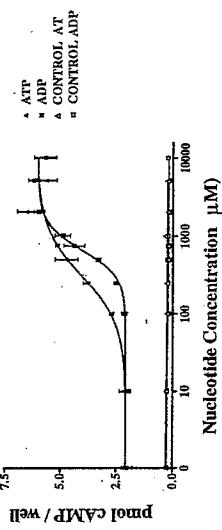


Fig. 3 Concentration curves of ATP and ADP on cAMP accumulation in TDAG8 expressing 293 cells. cAMP was measured after a 15 min. incubation with various nucleotides and analogues using the cAMP Flashplates (NEN). The data represents the mean +/- S.D. of triplicate experimental points.

CONCLUSIONS

- We have identified a conserved amino acid sequence at the carboxyl-terminal of transmembrane domain 3 as a landmark sequence for the P2Y purinergic receptors.

- The orphan G protein-coupled receptor, TDAG8, has such a conserved motif and is indeed an ATP/ADP receptor.

- TDAG8 is only coupled to Gs and is constitutively active. ATP and ADP activate TDAG8 further with EC₅₀ values of 310 μM and 660 μM, respectively, in transiently transfected 293 cells.

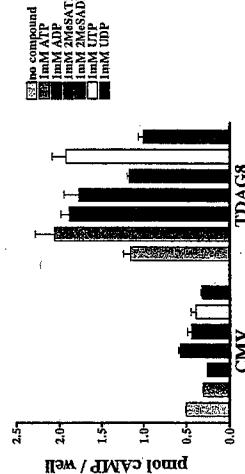


Fig. 2 cAMP accumulation in TDAG8 expressing 293 cells in response to nucleotides and nucleotide analogues. cAMP was measured after a 15 min. incubation with various nucleotides and analogues using the cAMP Flashplates (NEN). The data represents the mean +/- S.D. of triplicate experimental points.

Adenosine, an endogenous anti-inflammatory agent

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Cronstein, Bruce N. Adenosine, an endogenous anti-inflammatory agent. *J. Appl. Physiol.* 76(1): 5-13, 1994.—Adenosine receptors are present on most cells and organs, yet, although the physiological effects of adenosine were first described over 60 years ago, the potential therapeutic uses of adenosine have only been recognized and realized recently. A decade ago the potent anti-inflammatory effects of adenosine were first described; adenosine, acting at specific A_2 receptors, inhibits some, but not all, neutrophil functions. Adenosine inhibits phagocytosis, generation of toxic oxygen metabolites, and adhesion (to some surfaces and to endothelial cells) but does not inhibit degranulation or chemotaxis. Occupancy of adenosine A_2 receptors modulates leukocyte function by a novel mechanism. Although adenosine A_2 receptors are classically linked to heterotrimeric G_s signaling proteins and stimulation of adenylyl cyclase, adenosine 3',5'-cyclic monophosphate does not act as the second messenger for inhibition of leukocyte function. By a mechanism that still remains obscure, occupancy of adenosine A_2 receptors on neutrophils “uncouples” chemoattractant receptors from their stimulus-transduction proteins. The concentrations of adenosine that inhibit inflammatory cell function are similar to those observed in vivo and suggest a role for adenosine in the modulation of inflammation in vivo. Indeed, recent studies indicate that nonmetabolized adenosine receptor agonists are potent anti-inflammatory agents, and other studies indicate that methotrexate, a commonly used anti-inflammatory agent, diminishes inflammation by increasing adenosine release at inflamed sites. The observations reviewed here suggest that adenosine and agents that act through adenosine are excellent candidates for development as anti-inflammatory agents.

leukocyte; neutrophil; methotrexate; inflammation

INFLAMMATION IS RESPONSIBLE for tissue injury in pathological conditions ranging from myocardial infarction to rheumatoid arthritis. Recent studies suggest that adenosine is a potent regulator of the inflammatory response and, as such, may be useful as a pharmacological agent in the therapy of inflammatory disease. I review the effects of adenosine on leukocyte (primarily polymorphonuclear leukocytes) function and discuss the signal transduction mechanisms responsible for adenosine's effects on inflammatory cells and inflammation. Finally, I discuss the potential utility of adenosine and agents that act via adenosine as therapeutic modalities in controlling inflammation.

NEUTROPHILS, THE MOST ABUNDANT LEUKOCYTES, MEDIATE ACUTE INFLAMMATION

The histological determinant of acute inflammation is the presence of polymorphonuclear leukocytes (neutrophils). Neutrophils are the most abundant circulating leukocytes and are, generally, the first to respond to bacterial invasion or injury. To arrive at an inflamed site, neutrophils must first travel via the circulation, adhere

to the vascular endothelium of postcapillary venules, and finally migrate between the endothelial cells lining the vasculature and into the extravascular space. A variety of adhesion molecules expressed on both the surface of neutrophils and endothelium mediate this complex interaction. L-selectin is a member of the selectin family of lectinlike adhesion molecules that effects the initial loose contact of the resting neutrophil with the endothelium (rolling). When the leukocyte is further activated by tissue factors or bacterial products, L-selectin is shed from the neutrophil surface. Concurrently, other adhesion molecules, CD11a and CD11b/CD18 (β_2 -integrins) on the neutrophil and intracellular adhesion molecule 1 on the endothelial cell surface, mediate other more stable adhesive interactions between neutrophils and the endothelium. After adhesion to the vascular wall, neutrophils migrate into the inflamed or infected site by following a trail of chemoattractants, such as the surrogate bacterial chemoattractant *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), the activated complement component C5a, and the cytokine interleukin 8 (34).

Once in the extravascular space, the leukocytes phago-

cytose bacteria or debris, fuse the phagocytic vacuole with lysosomal granules (phagolysosomes), and digest or kill the ingested agents using a variety of granule enzymes (e.g., collagenase and elastase) and toxic oxygen metabolites (e.g., O_2^- and H_2O_2). Neutrophils may also injure tissues and organs by the overzealous secretion of these granule components and oxygen metabolites into the extracellular milieu. Recognition of the capacity of neutrophils to mediate tissue injury has driven the search for agents designed to specifically diminish leukocyte accumulation or function in the setting of myocardial injury and other acute inflammatory conditions.

Tissue mast cells, tissue macrophages, monocytes, and other cells also participate in the acute inflammatory response. Indeed, collaboration of these cells is essential for stimulating neutrophil-endothelial interactions and priming neutrophils so that they become more potent effector cells in the extracellular environment. On activation, mast cells release histamine, a potent vasodilator, which stimulates neighboring endothelial cells to express P-selectin. Tissue macrophages and monocytes release cytokines [e.g., interleukin 1 and tumor necrosis factor- α (TNF- α)], which provoke endothelial expression of E-selectin and intracellular adhesion molecule 1 and "prime" neutrophils, thereby permitting them to generate greater quantities of reactive oxygen metabolites in response to chemoattractants (34).

EFFECTS OF ADENOSINE ON NEUTROPHIL FUNCTION

In 1980 Marone et al. (69) observed that, unlike epinephrine, adenosine, an agent known to promote intracellular adenosine 3',5'-cyclic monophosphate (cAMP) accumulation in a variety of cell types, did not affect degranulation by stimulated neutrophils. These results were somewhat disappointing, and no further studies were performed to explore the effect of adenosine on neutrophil function for several years. In 1983 Cronstein et al. (28) reported that adenosine inhibited neutrophil O_2^- generation stimulated by chemoattractants (FMLP and C5a) and the Ca^{2+} ionophore A-23187 but not by phorbol myristate acetate (a direct activator of protein kinase C). This group also confirmed that adenosine was, at best, a poor inhibitor of granule release from stimulated neutrophils and did not inhibit leukocyte aggregation (neutrophil-neutrophil adhesion). Of note, adenosine mediated its effects on neutrophil function by acting extracellularly, since blockade of purine uptake did not reverse the effect of added adenosine on stimulated neutrophil function. Subsequent studies (33, 87) demonstrated that adenosine inhibited stimulated O_2^- generation via occupancy of specific adenosine receptors (A_2). The effects of adenosine on generation of oxygen radicals (O_2^- and H_2O_2) by activated neutrophils have now been reproduced in a number of laboratories (5, 10, 15, 35, 36, 47, 58, 74, 78, 94, 99, 113). In contrast to the original studies, some laboratories have reported that adenosine inhibits stimulated neutrophil degranulation and aggregation (86, 92, 95), whereas other laboratories could not demonstrate any great effect of adenosine on stimulated neutrophil degranulation or aggregation (11, 27, 45, 74, 110).

The effects of adenosine on chemotaxis, adhesion to

endothelial cells, and phagocytosis have subsequently been explored. Rose et al. (88) observed that adenosine and its analogues promoted chemotaxis to FMLP and C5a at concentrations two to three orders of magnitude below that which inhibited O_2^- generation. Cronstein et al. (30) first demonstrated that the stable adenosine analogue 2-chloroadenosine inhibited adhesion of stimulated neutrophils to endothelial cells, but subsequent studies by this same group have demonstrated that the effects of adenosine and its analogues on stimulated neutrophil adhesion to endothelial cells and other surfaces are more complex (31). As is discussed below, neutrophils, like other cell types, possess two different types of receptors for adenosine, A_1 and A_2 (23). Low concentrations of compounds specific for the adenosine A_1 receptor actually promote stimulated neutrophil adhesion to cultured endothelial cells and some surfaces. Higher concentrations of adenosine and adenosine A_2 -receptor-specific analogues inhibit stimulated neutrophil adhesion to endothelium. Similarly, low concentrations of adenosine A_1 -receptor agonists promote phagocytosis of immunoglobulin-coated red blood cells, whereas higher concentrations of adenosine or A_2 -receptor agonists inhibit phagocytosis of immunoglobulin-coated red blood cells (91).

After exposure to a variety of agents [e.g., phorbol myristate acetate, endotoxin (lipopolysaccharide), platelet-activating factor (PAF), TNF- α], neutrophils become primed to generate greater quantities of H_2O_2 and O_2^- after stimulation with chemoattractants. Adenosine has been reported to inhibit TNF- α -mediated priming for adherent neutrophils but not for neutrophils in suspension (36, 97). In contrast, Stewart and Harris (97) found that adenosine did inhibit PAF-mediated priming of neutrophils in suspension. When primed, neutrophils showed a diminished chemotactic response to chemoattractants and adenosine reversed the effect of TNF- α on neutrophil chemotaxis (98). The effects of adenosine on TNF- α priming of adherent neutrophils are qualitatively similar to those of adenosine and its agonists on TNF- α -mediated reduction of chemotaxis, since migrating cells must adhere to the underlying substratum (36, 97). Because priming is only poorly understood, the mechanism by which adenosine, acting on its receptor, inhibits priming remains a mystery.

One early observation that suggested a physiological role for adenosine as a regulator of inflammation was the demonstration that adenosine is released by suspensions of neutrophils or endothelial monolayers in vitro (10, 28, 30, 47, 77, 105). Removal of this endogenously released adenosine (by addition of adenosine deaminase) enhanced O_2^- generation by stimulated neutrophils. This finding led to the hypothesis that adenosine is an endogenously released anti-inflammatory agent, a "retaliatory metabolite" (28, 30, 77). Cronstein et al. (30) tested this hypothesis in vitro and found that stimulated neutrophils injured endothelial cells; endothelial cell injury was greatly enhanced when extracellular adenosine was removed by the addition of adenosine deaminase. Subsequent studies have confirmed that adenosine may function as an endogenous regulator of inflammation in vivo (19, 47). Indeed, recent studies have suggested that the anti-inflammatory effects of methotrexate, a commonly

TABLE 1. Effects of adenosine receptor occupancy on stimulated neutrophil function

	A ₁ Receptor Occupancy	A ₂ Receptor Occupancy
Oxygen radical generation (H ₂ O ₂ and O ₂ ⁻)	↔	↓
Chemotaxis	↑	↔
Phagocytosis	↑	↓
Adhesion	↑	↓

↑, Stimulates; ↓, inhibits; ↔, no effect.

used agent in the treatment of rheumatoid arthritis, result from enhanced adenosine release at inflamed sites (24, 32). A similar mechanism is probably responsible for the anti-inflammatory effects of acadesine (5-aminoimidazole-4-carboxamide ribonucleoside) (46), an agent currently under study for the prevention of reperfusion injury in patients undergoing cardiac surgery (see below).

In addition to neutrophils, other leukocytes also possess adenosine receptors. Adenosine inhibits generation of O₂⁻ (18, 64), phagocytosis of immunoglobulin-coated particles (90), and secretion of the complement component C2 (63) by stimulated monocytes. Probably much more central to the potential anti-inflammatory effects of adenosine is inhibition, by adenosine, of stimulated monocyte secretion of the proinflammatory cytokine TNF- α (82), although it is not known whether this effect is mediated by an adenosine receptor. Mast cells have previously been reported to possess adenosine receptors that stimulate release of such mediators as histamine (50, 70, 71), but subsequent studies have suggested that adenosine-mediated enhancement of mediator release is not mediated by a cell surface receptor, since cellular uptake is required for adenosine's functional effects (19, 20, 49, 66).

ADENOSINE RECEPTORS ON LEUKOCYTES: SUBTYPE AND SIGNALING

It was clear from even the earliest studies that adenosine interacted with a site on the cell surface to regulate neutrophil function. Metabolism of adenosine to inosine abrogated the effect of adenosine on neutrophil function, and, as noted above, adenosine uptake is not required for inhibition of neutrophil function (21, 28, 106). It had previously been discovered that other cell types possess two distinct receptors for adenosine designated A₁ and A₂ (67, 104). By using adenosine analogues specific for the individual receptors, it was demonstrated that adenosine interacted with an A₂ receptor to inhibit O₂⁻ generation by stimulated neutrophils (33, 87; Table 1). Similarly, adenosine interacted with A₂ receptors to inhibit stimulated neutrophil adhesion and phagocytosis of immunoglobulin-coated particles (31, 91).

Radioligand (2-[³H]chloroadenosine and 5'-N-[³H]-ethylcarboxamidoadenosine)-binding studies (using intact cells) demonstrated the presence of a single type of adenosine receptor on the surface of neutrophils with a dissociation constant of ~0.23 μ M (33). In this study it was estimated that there were ~11,000 receptors per neutrophil. Although interpretation of these studies was rendered more difficult by poor ligand specificity for A₂

receptors and the high nonspecific binding of the ligands used, subsequent ligand-binding studies (to isolated membranes) using a more specific ligand ([³H]CGS-21680) have generally confirmed these observations (72). Because of the high nonspecific binding of all of the ligands used, no evidence of a high-affinity A₁ receptor has been provided by radioligand-binding studies.

It quickly became clear that interaction with an A₂ receptor could not explain all of the effects of adenosine on neutrophil function; extremely low concentrations of adenosine or its agonists promoted adhesion and chemotaxis, and higher concentrations of adenosine or different agonists inhibited adhesion and generation of oxygen metabolites. This pharmacological profile of adenosine effects on neutrophil function is consistent with the presence of two distinct receptors that respond to different concentrations of adenosine (23, 31, 88). Thus, occupancy of adenosine A₁ receptors promotes phagocytosis and chemotaxis (half-maximal effective concentration in the picomolar range), whereas occupancy of A₂ receptors inhibits O₂⁻ generation and adhesion (half-maximal inhibitory concentration in the nanomolar range) (23, 31, 88). Although no radioligand-binding studies have yet demonstrated the presence of A₁ receptors on neutrophils, recent studies that used a monoclonal antibody directed against adenosine A₁ receptors confirmed the presence of adenosine A₁ receptors on the surface of human neutrophils (90).

SIGNAL TRANSDUCTION AT NEUTROPHIL ADENOSINE A₁ RECEPTORS

Adenosine receptors were first demonstrated and their subtypes differentiated on the basis of the effect of adenosine on cellular content of cAMP (67, 104). In cultured neural cells, A₁-receptor occupancy diminishes cAMP accumulation in response to other ligands (e.g., β -adrenergic agents) and occupancy of A₂ receptors directly stimulates the accumulation of cAMP. In a series of experiments performed in a number of laboratories, the susceptibility of adenosine A₁-receptor-mediated functions to inhibition by pertussis toxin [an agent that ADP-ribosylates and thereby inactivates inhibitory G (G_i) proteins] indicated that A₁ receptors were linked to G_i signal transduction proteins (3, 13, 37, 40, 43, 51, 75, 83-85, 89, 100). This observation was confirmed by the cloning of an A₁ receptor and its characterization as a member of the seven transmembrane-spanning family of G protein-linked receptors (103). In recent experiments it was found that, under conditions in which pertussis toxin only minimally affected chemotaxis to FMLP, pertussis toxin treatment reversed the effect of adenosine receptor occupancy on neutrophil chemotaxis (88). These results confirmed that the effect of adenosine on chemotaxis was mediated by A₁ receptors and indicated that, in neutrophils, A₁ receptors are similarly coupled to G_i proteins (88). In this regard it is interesting to note that the chemoattractant receptors of neutrophils are similarly linked to pertussis toxin-sensitive signal transduction systems (12, 14, 41, 59-61, 79-81, 96, 107-109). One can postulate that amplification of G_i-stimulated signals by A₁ agonists may account for their enhancement of che-

motaxis and phagocytosis. Alternatively, occupancy of A_1 receptors may promote more rapid recycling of chemoattractant and Fc receptors to effect more rapid chemotaxis and greater phagocytosis, respectively.

SIGNAL TRANSDUCTION AT NEUTROPHIL ADENOSINE A_2 RECEPTORS

Stimulus transduction at neutrophil adenosine A_2 receptors has been an area of great interest since the first description of the effects of adenosine on O_2^- generation. Adenosine A_2 receptors were first differentiated in neural cells on the basis of their capacity to stimulate intracellular accumulation of cAMP, presumably via stimulatory G protein (G_s)-linked stimulus transduction proteins (67, 104). Indeed, cloning of an adenosine A_2 receptor from dog thyroid confirmed that A_2 receptors are members of the family of seven transmembrane-spanning G protein-linked receptors as well (68). Like other G_s -linked receptors, adenosine receptors on neutrophils mediate the intracellular accumulation of cAMP (27, 62, 78). Moreover, occupancy of adenosine A_2 receptors enhances the increase in neutrophil cAMP that can be measured after stimulation with chemoattractants (27, 62). In general, accumulation of intracellular cAMP, either as a result of ligand-receptor interactions or treatment of neutrophils with cell-soluble cAMP analogues (dibutyryl cAMP), inhibits stimulated neutrophil functions, such as O_2^- generation. It seemed likely therefore that cAMP mediated inhibition of O_2^- generation by adenosine receptor ligation; however, there is little support for this hypothesis. Occupancy of adenosine receptors does not provoke detectable increases in neutrophil cAMP content except in the presence of a nonmethylxanthine phosphodiesterase inhibitor (Ro-20-1724), yet Ro-20-1724 does not increase the functional effect of adenosine receptor occupancy on stimulated generation of O_2^- (27). More recent studies also cast doubt on the hypothesis that cAMP is the intracellular messenger for inhibition of O_2^- generation by adenosine. Treatment of neutrophils with cell-soluble analogues of cAMP inhibits O_2^- generation, and, as would be expected, the inhibition of O_2^- generation by dibutyryl cAMP is completely reversed by inhibitors of the cAMP-dependent protein kinase (protein kinase A) (26). In contrast, the effects of adenosine A_2 receptor occupancy on O_2^- generation are totally unaffected by protein kinase A inhibitors (26). Thus, although cAMP can inhibit O_2^- production via its effects on protein kinase A, adenosine does not utilize the cAMP-protein kinase A system to inhibit O_2^- generation. It remains possible that other neutrophil functions are affected as a result of increased intracellular cAMP concentrations.

Stimulation of neutrophils with chemoattractants provokes increased phospholipid turnover with generation of diacylglycerol and inositol 1,4,5-trisphosphate, events closely tied to activation of G_i proteins. Cronstein and Haines (25) have reported that adenosine does not inhibit the early wave of diacylglycerol formation (15 s after stimulation). Similarly, Walker et al. (111) have

reported that adenosine receptor occupancy does not affect the FMLP-stimulated peak in inositol 1,4,5-trisphosphate, an increase observed 10 s after FMLP stimulation. Thus, adenosine does not inhibit the early signals that follow chemoattractant receptor occupancy. In addition to the early increase in diacylglycerol release, stimulated neutrophils also undergo a late sustained increase in diacylglycerol synthesis. In contrast to the early transient increase in diacylglycerol, occupancy of adenosine receptors diminished, by ~50%, the sustained increase in diacylglycerol that followed stimulation (25).

Chemoattractant-stimulated generation of inositol 1,4,5-trisphosphate leads to mobilization of intracellular Ca^{2+} ($[Ca^{2+}]_i$) stores and increments in free cytosolic Ca^{2+} in the neutrophil, as in other cell types. As adenosine receptor occupancy does not affect the early events after occupancy of chemoattractant receptors, it was not surprising that the initial peak in free cytosolic Ca^{2+} was unaffected by treatment with adenosine analogues (27, 112). The observation that adenosine receptor occupancy does not affect either chemoattractant-stimulated alterations in phospholipids or rapid-onset fluxes in $[Ca^{2+}]_i$ metabolism suggests that adenosine does not interfere with the early steps in cell activation. Moreover, as neutrophil degranulation is much more tightly linked to the early stimulated changes in $[Ca^{2+}]_i$, the lack of an effect of adenosine on $[Ca^{2+}]_i$ is consistent with previous observations that adenosine does not inhibit degranulation.

Although adenosine does not affect the early increment in free cytosolic Ca^{2+} in stimulated neutrophils, Ward et al. (112) and Thiel and Bardenheuer (99) have observed that adenosine inhibits the sustained increase in free cytosolic Ca^{2+} that follows stimulation. Laghi Pasini et al. (62) have observed similar changes in free cytosolic Ca^{2+} and have further reported that adenosine is a Ca^{2+} -channel blocker, since adenosine inhibits binding of flunarizine, a Ca^{2+} -channel blocker, to neutrophil plasma membrane sites, a finding largely confirmed by Tsuruta et al. (101). Because the effect of adenosine on A-23187-stimulated O_2^- formation (chemiluminescence) could be reversed by addition of excess Ca^{2+} to the medium, Laghi Pasini et al. concluded that adenosine inhibits production of O_2^- by inhibiting stimulated Ca^{2+} influx or mobilization. Moreover, Thiel and Bardenheuer (99) observed that chelation of intracellular and extracellular Ca^{2+} reversed the effect of adenosine on the generation of O_2^- stimulated by insoluble particles (latex beads); the effect of adenosine on O_2^- generation by cells in which only the intracellular Ca^{2+} had been chelated was not tested. In contrast, Cronstein et al. (27) observed that adenosine inhibited chemoattractant-stimulated O_2^- generation even in the absence of extracellular Ca^{2+} . Although it is difficult to reconcile all of the experimental differences, it is clear that the signals for generation of O_2^- generation differ depending on whether the stimulus is a particle (e.g., latex particles or opsonized red blood cells), an ionophore (A-23187), or a chemoattractant (FMLP). Thus, although it is likely that adenosine, acting at its receptor, inhibits a Ca^{2+} -dependent step in neutrophil activation, it is by no means clear that adenosine

inhibits neutrophil function by inhibiting mobilization or transmembrane fluxes of Ca^{2+} . It is interesting to note that inhibition by adenosine of the sustained increase in $[\text{Ca}^{2+}]_i$ parallels the inhibition by adenosine of the sustained increase in diacylglycerol synthesis.

Whether adenosine acts as a Ca^{2+} -channel blocker or inhibits a Ca^{2+} -dependent step in stimulus transduction, recent studies indicate that adenosine receptors inhibit a proximal step in signal transduction for chemoattractant receptors. Adenosine receptor occupancy does not affect the capacity of NaF, an agent that directly activates G proteins, to stimulate O_2^- generation, an observation that suggests that adenosine inhibits the interaction between occupied chemotactic receptor and G proteins (15, 23). Moreover, adenosine A_2 -receptor (and β -adrenergic receptor) occupancy, but not cAMP or dibutyryl cAMP, inhibits FMLP-stimulated G protein activation, determined as the FMLP-stimulated increment in guanosine-triphosphatase activity (26). This observation was recently confirmed by Burkey and Webster (15). These data indicate that adenosine receptor occupancy either inhibits or uncouples bound FMLP receptors from signal transduction mechanisms.

A separate line of evidence suggests a possible mechanism by which adenosine receptor occupancy can uncouple FMLP receptors from signal transduction mechanisms. Once occupied by ligand, FMLP receptors assume a high-affinity configuration for FMLP and associate with the cytoskeleton (16, 38, 53–56, 114). Subsequently, these bound receptors are no longer capable of stimulating neutrophil function. Occupancy of adenosine receptors promotes more rapid and complete association of bound FMLP receptors with the cytoskeleton (22, 23). Disruption of actin filaments (by cytochalasin B) diminishes the number of FMLP receptors that are associated with the cytoskeleton, disrupting the normal “turn-off” process, and thereby amplifying the FMLP-stimulated generation of O_2^- (55). Adenosine A_2 -receptor occupancy promoted the association of bound FMLP receptors to cytoskeletal components even in the presence of cytochalasin B and appeared to do so without affecting either basal or FMLP-stimulated actin filament formation (22). Thus adenosine restores, or augments, the regulatory mechanisms that dampen O_2^- generation. In contrast to these observations, Tsuruta et al. (102) have observed that adenosine and its analogues diminished filamentous actin content in both stimulated and resting neutrophils after long periods of incubation (15 min). The effect of adenosine on stimulated actin filament formation observed by Tsuruta et al. varied with the stimulus; adenosine receptor occupancy inhibited FMLP-stimulated actin filament formation only minimally but was a potent inhibitor of PAF-stimulated actin filament formation. Taken together these observations suggest a more general mechanism whereby adenosine A_2 -receptor occupancy inhibits chemoattractant-stimulated O_2^- generation: adenosine A_2 receptor occupancy promotes more rapid desensitization of chemoattractant receptors by enhancing both the rate and the extent to which bound chemoattractant receptors associate with the cytoskeleton.

SIGNAL TRANSDUCTION AT NEUTROPHIL ADENOSINE RECEPTORS, A SUMMARY

Although adenosine A_2 receptors were first differentiated from A_1 receptors by the capacity of bound A_2 receptors to stimulate an increase in intracellular cAMP, this cyclic nucleotide does not appear to play a role in the inhibition by adenosine of O_2^- generation. Similarly, although adenosine A_2 -receptor occupancy diminishes the late increment in $[\text{Ca}^{2+}]_i$ by, presumably, blocking Ca^{2+} uptake, the role of Ca^{2+} as the intracellular message inhibited by adenosine receptor occupancy is not clear. The precise signaling events for adenosine A_2 receptors in the neutrophil have yet to be worked out, but they provide a notable exception to the rule that adenosine receptor occupancy modulates cellular function via cAMP.

ADENOSINE INHIBITS INFLAMMATION: IN VITRO AND IN VIVO STUDIES

Adenosine is present in whole blood (in humans) at a concentration of ~ 300 nM (see Ref. 48), and recent animal studies demonstrate higher adenosine concentrations in ischemic (as high as 1,200 nM) or inflamed (~ 5 –600 nM) tissues (32, 73). Cronstein et al. (32) and Newby et al. (77) first demonstrated that suspensions of neutrophils release adenosine into the extracellular milieu. Removal of this endogenously released adenosine led to a significant increase in neutrophil responses to chemoattractants (28). Subsequent studies have confirmed these early observations (105). Other cells may release adenosine into the extracellular milieu, such as vascular endothelium. In 1986 it was first demonstrated that both an exogenously applied adenosine receptor agonist (2-chloroadenosine) and endogenously released adenosine protected human vascular endothelial cells from injury by stimulated human neutrophils (30). These observations have subsequently been confirmed by Gunther and Herring (47), and adenosine has been suggested to be a critical regulator of inflammation. Moreover, these results suggested that increased adenosine release could be utilized to diminish inflammation. Interestingly, the existence of a potential counterregulatory effect of extracellular adenosine deaminase (released from dead cells or bacteria) was inferred from the observation that adenosine deaminase can bind to opsonized particles (zymosan) and, by metabolizing extracellular adenosine to inosine, may promote phagocytosis and O_2^- generation by activated leukocytes (29).

Adenosine and its analogues are potent inhibitors of inflammation in two different animal models (44, 57, 93). Green et al. (44) have reported that a single daily dose (intraperitoneal) of adenosine reduced the severity of experimental adjuvant arthritis in rats, a finding that is quite surprising considering the extremely rapid metabolism of adenosine [half time in whole blood is 2 s (76)]. Although adenosine diminishes leukocyte function via A_2 receptors and, in general, promotes inflammatory functions via A_1 receptors, Schrier and co-workers (65, 93) have observed that adenosine A_1 -receptor agonists are better inhibitors of pleural and peritoneal inflammation than A_2 -receptor agonists when studied *in vivo*. It is not

clear whether the apparent A₁-receptor specificity is a result of individual characteristics of the agonists used, which, although possessing higher affinity for A₁ than A₂ receptors, are not absolutely specific. Indeed, subsequent studies using adenosine receptor antagonists that are highly specific for their subtype suggested that adenosine modulates inflammation in vivo via A₂ receptors (4).

Further in vitro studies suggested that pharmacologically enhanced release of endogenous adenosine may already be utilized to diminish inflammation (24). Methotrexate is an antifolate that is commonly administered in low doses at weekly intervals to treat such inflammatory diseases as rheumatoid arthritis. Methotrexate is rapidly polyglutamated. The polyglutamated derivatives accumulate in cells and tissues where they may still actively inhibit folate-dependent enzymes (1, 7, 17). Among other effects, methotrexate promotes the accumulation of dihydrofolate polyglutamates (1, 2). Both dihydrofolate polyglutamates and methotrexate polyglutamate inhibit the enzyme 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase, leading, potentially, to intracellular accumulation of AICAR (1, 2). The intracellular accumulation of AICAR enhances adenosine release from stressed cells (8, 9), which leads to the interesting hypothesis that adenosine may actually be the mediator of the anti-inflammatory effects of methotrexate. Cronstein et al. (24) have tested this hypothesis in an in vitro system and observed that treatment of endothelial cells or fibroblasts with methotrexate led to a modest increase in adenosine release. However, a much greater increase in adenosine release was observed when the methotrexate-treated cells were exposed to a stress (in this case activated neutrophils). Fewer stimulated neutrophils adhered to the methotrexate-treated endothelial cells and fibroblasts in this in vitro model of inflammation, and the diminished neutrophil adhesion was due to the increase in adenosine release from the methotrexate-treated cells. A similar increase in adenosine release and an adenosine-mediated decrease in inflammatory cell-to-cell interactions were observed when fibroblasts and endothelial cells were treated with AICARiboside, a cell-soluble precursor of AICAR. Recent studies by Asako et al. (4) confirmed this hypothesis in vivo; methotrexate applied topically (and in relatively high concentration) diminished leukocyte extravasation from the vasculature via a mechanism that is dependent on the presence of adenosine; adenosine deaminase reversed the anti-inflammatory effect of methotrexate. Moreover, using specific antagonists, these investigators demonstrated that the anti-inflammatory effects of methotrexate, acting via adenosine, were due to interaction with adenosine A₂ receptors (presumably on the leukocytes). More recently it was demonstrated that weekly treatment of mice with pharmacologically relevant doses of methotrexate increased splenocyte AICAR content, increased adenosine concentration in inflammatory exudates, and inhibited leukocyte accumulation in inflammatory exudates (murine air pouch model of inflammation) by an adenosine A₂-receptor-mediated effect (32). Similarly, Gruber et al. (46) have shown that intravenous infusions of AICARiboside also diminished leukocyte accumulation and cardiac injury in a model of ischemic injury (reperfusion

injury), although the role of adenosine in the inhibition of cardiac injury is less clear in this model. Because sulfasalazine, an anti-inflammatory agent developed to treat rheumatoid arthritis and more commonly used to treat inflammatory bowel disease, may also interfere with AICAR metabolism (44), Baggott et al. (6) have suggested that sulfasalazine may diminish inflammation via adenosine release in a manner similar to methotrexate.

ADENOSINE RECEPTORS AND INFLAMMATION, A LOOK INTO THE FUTURE

Strong evidence from in vitro and in vivo studies indicates that adenosine can act as a potent inhibitor of inflammation. The medicinal use of adenosine is limited by its rapid metabolism and by its potent cardiovascular effects; however, it is possible that new, more highly specific agonists may be developed that can diminish inflammation without the unwanted side effects (hypotension, bradycardia) experienced by individuals treated with adenosine. More recently attention has been directed at developing agents that promote the release of adenosine at inflamed sites for use in the treatment of inflammatory diseases. Even now the therapeutic potential of enhanced release of adenosine is being tested in the clinic; trials are currently underway on the use of AICAR in the prevention of cardiac injury during coronary artery bypass grafting. Recent preliminary studies in animals indicate that adenosine kinase inhibitors, presumably by enhancing adenosine release at inflamed sites, may also be potent anti-inflammatory and antirheumatic agents (39). If clinical trials with AICAR prove the efficacy of this agent, then adenosine, adenosine receptor agonists, and agents that promote adenosine release at sites of inflammation will provide fertile ground for development of new, safer, and more effective anti-inflammatory agents.

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Adenosine Physiology and Pharmacology: How About A₂ Receptors?

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ABSTRACT. Adenosine participates in the physiology of central and peripheral tissues through several subtypes of G-protein-coupled receptors. Positively linked to adenylyl cyclase, A₂ receptors have been subdivided into A_{2a} and A_{2b} sites on the basis of their molecular, biochemical and pharmacological properties. They exhibit selective distribution, and are implicated in the modulation of psychomotor activity, circulation, respiration, and metabolism. Recent data support the evidence that adenosine A₂ receptor properties may prove useful in future drug development, and selective manipulation of receptor-associated biologic effects might be relevant in the treatment of various disorders, including psychiatric diseases, hypoxia/ischemia, inflammation or erythrocytosis. Copyright © 1996 Elsevier Science Inc. PHARMACOL. THER. 71(3): 325-335, 1996.

KEY WORDS. Adenosine, A₂ receptors, biochemical properties, maturation, central and peripheral physiology, therapeutic potential.

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ABBREVIATIONS. CGS 21680, 2-[*p*-(2-carboxyethyl)-phenylethylamino]-5'-N-ethylcarboxamidoadenosine; GABA, γ -aminobutyric acid; NECA, 5'-(*N*-ethylcarboxamido)adenosine.

1. INTRODUCTION

The nucleoside adenosine is a widely distributed ubiquitous biologic compound that mediates a variety of physiological responses in mammalian systems (Daval *et al.*, 1991; Collis and Hourani, 1993; Fredholm, 1995). Its pharmacological effects are mediated by specific receptors initially classified into A₁ and A₂ receptors on the basis of their respective affinity for the nucleoside and upon structural and pharmacological criteria, which include inhibition (A₁) or stimulation (A₂) of adenylyl cyclase activity (van Calker *et al.*, 1979). More recently, molecular cloning has led to the identification of four distinct adenosine receptor subtypes: namely, A₁, A_{2a}, A_{2b} and A₃, which display specific properties (Fredholm *et al.*, 1994).

Whereas A₁ receptors have been studied extensively, their implication in the neuromodulatory and cardiovascular effects of adenosine has been well established (Dunwidie and Fredholm, 1989; Belardinelli *et al.*, 1989). In this

respect, numerous reviews have focused on A₁ receptor ligands as potential therapeutic entities in various pathological states, e.g., hypoxia/ischemia, stroke, seizures, psychiatric disorders, nociception, as well as cardiac and coronary artery diseases (Daval *et al.*, 1991; Williams, 1993; Deckert and Gleiter, 1994).

Due to the recent development of more specific tools for studying A₂ receptors, there is now growing interest in the physiological and pharmacological role of adenosine specifically mediated by the high- and low-affinity A₂ receptor categories, i.e., A_{2a} and A_{2b} (Bruns, 1980; Daly *et al.*, 1983; Bruns *et al.*, 1986).

2. RECEPTOR CHARACTERIZATION

Direct identification and characterization of A₂ receptors have been made possible by the synthesis of selective radioligands, mainly the agonist [³H]2-[*p*-(2-carboxyethyl)-phenylethylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680) that binds with high affinity in a variety of tissues to specific sites with pharmacological properties expected of the adenosine A₂ receptor (Jarvis *et al.*, 1989; Wan *et al.*, 1990), whereas the drug potency is 140 times weaker at A₁ recep-

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tors (Hutchison *et al.*, 1989). The use of CGS 21680 and other 2-substituted adenosine analogues allows the discrimination between A_{2a} receptor subtype with high affinity for CGS 21680 and A_{2b} receptor subtype with much lower affinity for this compound (Hutchison *et al.*, 1990; Gurden *et al.*, 1993). In addition, binding and functional assays have shown that (E)-1,3-dialkyl-7-methyl-8-(3,4,5-trimethoxy-styryl)xanthines are selective antagonists at A_2 receptors (Shimada *et al.*, 1992). Another xanthine derivative, 8-(3-chlorostyryl)caffeine, is highly selective for A_{2a} receptors, both *in vitro* and *in vivo* (Jacobson *et al.*, 1993), while a non-xanthine compound, 4-(2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol, recently has been described as a very potent and selective A_{2a} antagonist (Poucher *et al.*, 1995). To date, 5'-(N-ethylcarboxamido)adenosine (NECA), the compound previously used for the analysis of A_2 receptors (Bruns *et al.*, 1986), is considered the most selective agonist for A_{2b} receptors (Hide *et al.*, 1992), whereas currently no specific antagonist is available. Finally, as for A_1 receptors and by contrast with A_3 receptors, the worldwide ingested caffeine and theophylline are inhibitors at both A_{2a} and A_{2b} subtypes.

A_2 receptors are glycoproteins with molecular mass around 45 kDa (Barrington *et al.*, 1990). They have been cloned from various species, including rat, guinea pig, dog and human (Libert *et al.*, 1989; Maenhaut *et al.*, 1990; Fink *et al.*, 1992; Pierce *et al.*, 1992; Salvatore *et al.*, 1992; Stehle *et al.*, 1992; Meng *et al.*, 1994). A highly conserved homology in the amino acid sequence has been reported between A_{2a} and A_{2b} subtypes. The receptor entities are quite similar in their transmembrane parts, but differ in the carboxy terminal domain, with a larger tail for the A_{2a} receptor, the functional significance of which remains unclear (see Fredholm *et al.*, 1994).

Like other adenosine receptors, A_2 subtypes belong to the G-protein-coupled receptor family. Due to their ability to stimulate adenylate cyclase, they are expected to be linked to Gs-proteins. However, although it is still not definitely established whether other G-proteins interact with A_2 receptors, whether their associated Gs-proteins can activate other effector systems, and whether A_{2a} and A_{2b} receptor subtypes display different signaling characteristics, recent studies have shown that both A_{2a} (Kirk and Richardson, 1995) and A_{2b} (Mateo *et al.*, 1995) receptors would likely exert some of their effects by altering calcium fluxes. In the striatum, the activation of A_{2a} receptors by CGS 21680 inhibits γ -aminobutyric acid (GABA) release. Such an action appears to be cyclic AMP-independent, and would involve an inhibition of calcium influx through N-type channels via a pertussis toxin-insensitive G-protein, or possibly by inhibition of protein kinase C that may itself affect subsequently N-type calcium channels (Kirk and Richardson, 1995). Likewise, the A_{2b} receptors present on the plasma membrane of chromaffin cells have been suggested to inhibit secretagogue-evoked catecholamine release by activation of a protein phosphatase and inhibition of calcium influx pathway (Mateo *et al.*, 1995).

3. RECEPTOR DISTRIBUTION

Adenosine receptors exhibit a fairly wide distribution in the body. Within the brain, A_{2a} receptors have been shown to be highly concentrated in regions that encompass caudate putamen, globus pallidus, nucleus accumbens and olfactory tubercle. These observations repeatedly have been reported by means of radioligand binding techniques in membrane preparations (Jarvis *et al.*, 1989; Wan *et al.*, 1990; Mazzoni *et al.*, 1993) or by autoradiography in brain sections using a tritiated ligand, i.e., [3 H]CGS 21680 (Jarvis and Williams, 1989; Parkinson and Fredholm, 1990; Meng *et al.*, 1994), or a radioiodinated ligand, i.e., [125 I]2-[2-(4-azido-3-iodophenyl)ethylamino]adenosine (Luthin *et al.*, 1995). Accordingly, abundant expression of A_{2a} mRNA was shown in the striatum (Schiffmann *et al.*, 1991b; Fink *et al.*, 1992). Striatal A_{2a} receptor mRNA has been found exclusively in a subpopulation of medium-sized spiny neurons that coexpress enkephalin (Schiffmann *et al.*, 1991b) and dopamine D₂ receptors (Fink *et al.*, 1992). These striatal neurons are known to project to the globus pallidus and utilize GABA as a neurotransmitter (Gerfen, 1992). By contrast, A_{2a} receptors were found very sparsely in neurons projecting to the substantia nigra that express dopamine D₁ receptors and substance P (Schiffmann and Vanderhaeghen, 1993; Pollock *et al.*, 1993). Due to the demonstration of an A_{2a} receptor-mediated stimulation of acetylcholine release from rat striatal synaptosomes, the presence of these receptors was reported on cholinergic terminals (Kirk and Richardson, 1994), although Schiffmann and co-workers (1991a) failed to show any expression of adenosine A_{2a} receptor mRNA in neurons containing choline acetyltransferase transcript. On the other hand, although at a lower level than in the striatum, [3 H]CGS 21680 specific binding has been reported to membranes isolated from cerebral cortex (Wan *et al.*, 1990; Ji *et al.*, 1992), superior colliculus (Wan and Geiger, 1990) and hippocampus (Cunha *et al.*, 1994), and the receptor agonist would also exert functional activity in brain areas outside the striatum (Phillip, 1990; Sebastiao and Ribeiro, 1992; Cunha *et al.*, 1994). In agreement with such data, the expression of A_{2a} mRNA was reported in rat cortex and hippocampus (Fink *et al.*, 1992; Cunha *et al.*, 1994). In a recent autoradiographic study, Johansson and Fredholm (1995) have shown that the order of potency of a series of selective agonists and antagonists to displace [3 H]CGS 21680 binding, as well as the strong magnesium-dependency of the agonist binding, in rat hippocampus and cerebral cortex contrast with the properties expected for A_{2a} receptors, and even with those expected for A_{2b} and A_1 receptors. The authors concluded that in these brain regions, and possibly other structures, [3 H]CGS 21680 likely binds to a site different from the classical striatal A_{2a} receptor and thus still unidentified adenosine receptor subtype. A_{2a} receptor mRNA has been also found in human heart, kidney and lung (Linden *et al.*, 1993), as well as in animal spleen (Meng *et al.*, 1994), while pharmacological and biochemical studies have shown the presence of A_{2a} receptors in liver (Johnson, 1982) and platelets (Hüttemann *et al.*, 1984).

Adenosine A_{2b} receptors have been only poorly characterized. They would be distributed more unevenly throughout the brain (Bruns *et al.*, 1986), and have been identified in the hypophyseal pars tubularis of the pituitary gland (Stehle *et al.*, 1992). Northern blot analysis has demonstrated expression of A_{2b} receptor transcript in brain and spinal cord, as well as in lung, caecum, large intestine and urinary bladder (Stehle *et al.*, 1992). Recently, a polyclonal antibody to the human A_{2b} receptor has been produced, and Western blotting has revealed the presence of the receptor in a variety of tissues, including thymus, colon, small intestine and placenta (Puffinbarger *et al.*, 1995).

4. RECEPTOR ONTOGENY

Accumulating evidence indicates that adenosine plays an important physiological role during development, as well as in regard to neonatal adaptation (Berne, 1986; Lagercrantz, 1994). Neonates exhibit a significant deficiency in adenosine brain levels at basal conditions, with substantial changes occurring shortly after birth. The lower concentration of the nucleoside in part would be due to an increased degradation of adenosine around birth. Nevertheless, the newborn can produce adenosine amounts similar to those of the adult under pathophysiological conditions (Aranda *et al.*, 1989). Even though it is well known that the density of adenosine receptors, as well as their functional capacity, are critical factors controlling the strength of adenosine action, only few studies have focused on developmental aspects of adenosine A₂ receptors. While embryonic kidney cells *in vitro* have been shown to express A_{2a} receptors positively linked to adenylyl cyclase (Furlong *et al.*, 1992), Schiffmann and Vanderhaeghen (1992) have examined the ontogeny of A_{2a} receptor mRNA and CGS 21680 binding sites by *in situ* hybridization and receptor autoradiography in pre- and postnatal rat striatum, postnatal dog striatum and in human fetus striatum. They observed an early expression of A_{2a} receptor mRNA and binding sites, with heterogeneous distributions and patches of dense labeling corresponding to dopamine D₁ and mu-opiate receptor-enriched regions. It suggested a role for A_{2a} receptors in the generation, maturation, and/or maintenance of the striatal compartmentalization. In another study, the expression of A_{2a} receptor mRNA was found in the developing rat striatum as early as day 14 of gestation, with some developmental alterations in the pattern of gene location within the striatum, which would indicate that the receptor mRNA is expressed by striatal neurons soon after their complete neurogenesis (Weaver, 1993). The same study also documented a transient expression of A_{2a} receptor mRNA in other brain areas than striatum, including cerebral cortex, subiculum, thalamic nuclei, locus caeruleus, area postrema, intermediate lobe of the pituitary gland, as well as in the cerebral vasculature, but extrastratal A_{2a} receptor mRNA could be detected only during the fetal period, with a peak on day 20 of gestation. The author postulated that such a transient expression would reflect the trophic influence of

adenosine on brain development through interaction with its A_{2a} receptors. We also analyzed the postnatal brain maturation of [³H]CGS 21680 binding to specific A_{2a} receptors in rat striatal membrane preparations and by quantitative autoradiography in brain sections (Doriat *et al.*, 1996). At birth, adenosine A_{2a} receptor levels correspond to about 3% of the adult density. They appeared to be concentrated in the striatum at all ages, with minor changes in the expression pattern within the various striatal regions, i.e., caudate putamen, nucleus accumbens, globus pallidus, olfactory tubercle. Interestingly, saturation experiments revealed a single population of sites throughout the postnatal period and showed a sharp decrease in the receptor affinity during brain development, K_d values rising from 2 to 15.5 nM between birth and adulthood. In addition, binding studies performed in the presence of a GTP analogue, guanylyl-5'-imidodiphosphate, led to the conclusion that A_{2a} receptors are linked to their G-proteins as soon as birth, although the coupling was weaker at birth than in more mature striatum. Taken together, the developmental studies emphasize the maturational role that adenosine may play via its specific A_{2a} receptors. In this respect, it has been shown that adenosine stimulates astrocyte proliferation *in vitro* via A₂ receptor activation (Rathbone *et al.*, 1991).

Very little is known about the development of A_{2b} receptors. In their immunohistochemical study, Puffinbarger and co-workers (1995) reported reactivity with the anti-A_{2b} receptor peptide antibody in mouse fetus *in utero* on day 15 of gestation. Strong specific immunoreactivity was apparent in the giant trophoblast and spongiotrophoblast cells comprising the basal zone of the murine chorioallantoic placenta. Cell labeling was also observed in multiple regions of the fetal nervous system, including cerebral cortex and septum, spinal cord, as well as spinal and autonomic ganglia. The authors concluded that the distribution of the A_{2b} receptor is consistent with pleiotropic roles for this receptor subtype during embryogenesis.

5. PHYSIOLOGICAL ROLES OF A₂ RECEPTORS

Via interaction with its specific receptors, adenosine is known to mediate a variety of critical physiological functions.

5.1. Behavior

At the behavioral level, adenosine and related substances can induce hypoactivity, depression of spontaneous and/or evoked locomotor activity, sedation, motor incoordination, ataxia, anticonvulsant actions and analgesia (see Barraco, 1991). Drugs that elevate endogenous levels of cyclic AMP, such as forskolin or rolipram, a phosphodiesterase inhibitor, can mimic many of the behavioral actions of adenosine, suggesting the participation of A₂ receptors (Wachtel, 1983; Barraco *et al.*, 1985). Accordingly, in an extensive series of experiments, NECA and CGS 21680 were found to be the most potent compounds in depressing locomotor activity, whereas specific A₁ receptor ligands were virtually

devoid of action (Phillis et al., 1986; Griebel et al., 1991; Janusz and Berman, 1992), and data are consistent with a central mediation of NECA-induced hypomotility (Durcan and Morgan, 1989). Very recently, Jain et al. (1995) have investigated the effects of specific purine analogues on mouse anxiety behavior by use of the elevated plus-maze, i.e., a mild anticonflict paradigm with self-exposure to a novel, potentially dangerous environment. Their results show that while the selective activation of central A_1 receptors produces anxiolytic-like behavior, activation of A_2 sites has no effect on plus-maze behavior, but suppresses the consequences of activating A_1 receptors, suggesting that A_2 receptors may be involved in adenosine-related anxiety behavior, as well as in the well-known anxiogenic-like activity of caffeine. Since A_{2a} receptors are highly concentrated within dopamine-enriched brain areas, several authors have discussed the involvement of adenosine in the physiology of striatum, where dopamine plays a prominent role in behavioral functions. The colocalization of adenosine A_{2a} and dopamine D_2 receptors, as mentioned in Section 3, constitutes the anatomical basis for their functional interactions evoked in behavioral studies (Ferré et al., 1992). Numerous reports suggest that central dopaminergic neurotransmission is involved in the mediation of the psychomotor effects of adenosine and methylxanthines. In rats, adenosine analogues inhibit the locomotor activity induced by dopamine agonists, whereas methylxanthine-induced stimulation of locomotor activity can be antagonized by dopamine depletion or D_2 receptor blockade. Likewise, it has been demonstrated that activation of A_{2a} receptors induces catalepsy, which can be counteracted by adenosine antagonists or by dopamine agonists (Ferré et al., 1991b). In membrane preparations of rat striatum, the A_{2a} receptor agonist CGS 21680 has been shown to decrease the binding of dopamine agonists to D_2 receptors, without affecting the binding to D_1 receptors (Ferré et al., 1991b, 1992). Evidence has been provided that adenosine may selectively modulate dopamine-mediated mesolimbic behavioral circuits via agonist actions at A_{2a} receptors in the nucleus accumbens (Barraco et al., 1994). On the other hand, in accordance with the location of adenosine A_{2a} binding sites in GABAergic neurons (Schiffmann and Vanderhaeghen, 1993), activation of adenosine receptors augments electrically evoked release of GABA from globus pallidus slices, reflecting the modulation by adenosine of GABAergic outputs from the striatopallidal efferent system (Mayfield et al., 1993).

5.2. Cardiovascular System

Pronounced cardiac and vascular effects of adenosine have been established for many years. However, it has been particularly difficult to demonstrate the involvement of selective receptor subtypes, especially due to the necessity to perform *in vivo* studies. NECA administration to rodents has been shown to reduce both blood pressure and heart rate, while CGS 21680 decreases blood pressure, with the concomitant appearance of a reflex tachycardia. Major ade-

nosine effects on the heart, such as the negative dromotropic and inotropic effects, have been attributed to activation of A_1 receptors (Belardinelli et al., 1989), and the physiological role of A_2 receptors present in atrial and ventricular myocytes remains unclear (Liang, 1992). More important are the A_2 receptor-mediated vascular effects of adenosine. The nucleoside is a potent coronary vasodilator, capable of relaxing the small arteries or arterioles, as well as the larger conductance vessels, such as the epicardial coronary artery and aorta rings. The relaxant action of adenosine on the vasculature would be mediated by A_2 receptors located on both the endothelial cells and the underlying vascular smooth muscles (Haedrick and Berne, 1990). In this respect, a mechanistic model has been proposed to explain the role of vascular receptors in mediating the vasodilatory action of adenosine (Liang, 1992). Under physiological conditions, vascular relaxation is produced by endothelial A_2 receptors that cause endothelium-dependent relaxation. When the energy demand is increased, e.g., following hypoxia/ischemia, large amounts of adenosine are released both from the endothelial cells into the coronary circulation and from the cardiac and vascular myocytes into the interstitium, and A_2 receptors of the endothelium and the vascular smooth muscle cells, respectively, mediate the endothelium-dependent and -independent vasodilatation. In the guinea pig coronary vasculature *in vitro*, Vials and Burnstock (1993) have reported that a subpopulation of A_2 receptors on the endothelial cells would mediate relaxation via production of the bioradical nitric oxide. To date, the functional subdivision of the A_{2a} and A_{2b} receptors in vascular beds is still unclear, but the order of potency of adenosine analogues for relaxing isolated rat aorta by an endothelium-dependent mechanism suggests the main participation of A_{2a} receptors (Lewis et al., 1994). Involvement of A_{2a} receptors has been also shown in the bovine, porcine and guinea pig coronary arteries, as well as in the rabbit aorta, mesenteric and coeliac arteries, whereas vasodilator effects of adenosine would be achieved through A_{2b} receptors in the guinea pig aorta and the dog saphenous vein (Collis and Hourani, 1993). Adenosine A_{2a} receptors also mediate the inhibition of platelet aggregation through the generation of cyclic AMP (Huttmann et al., 1984; Dionisotti et al., 1992), the A_2 receptor of human platelet exhibiting binding properties similar to the striatal A_{2a} receptors (Lohse et al., 1988).

Adenosine has been recognized as a powerful regulator of cerebral circulation (Phillis, 1989). Adenosine-induced relaxation of vascular smooth muscle results in a fall in cerebrovasculature resistance and thus, in an increase in cerebral blood flow. However, only sparse information is available concerning the effects of adenosine on cerebral blood flow, with results somewhat controversial. Adenosine infusion into the carotid artery in baboons produced a significant increase in global cerebral blood flow (Forrester et al., 1979), whereas adenosine injected via the carotid artery at various concentrations remained without effect on blood flow in the cerebrum, brainstem and cerebellum in the dog (Heistad et al., 1981). The latter data may be explained by

the rapid breakdown of adenosine injected. The influence of adenosine analogues on rat local cerebral blood flow was then investigated in two separate studies by means of the [¹⁴C]iodoantipyrine method (McBean *et al.*, 1989) or by the hydrogen clearance technique (Van Wylen *et al.*, 1989). In the first study, adenosine or its analogues were infused via the right internal carotid artery; adenosine itself (3.10^{-7} mol/min) and the A₂ receptor agonist NECA (10^{-1} mol/min) increased regional cerebral blood flow, while A₁ receptor agonists (10^{-9} – 10^{-10} mol/min) decreased it. Conversely, in the second study, local infusion of adenosine (10^{-3} – 10^{-5} M) and all its analogues (10^{-4} – 10^{-7} M) increased cerebral blood flow, with a more sensitive response to NECA. Recently, we analyzed the effects of the i.v. acute administration of more selective adenosine receptor ligands on local cerebral blood flow in the freely moving rat by the [¹⁴C]iodoantipyrine autoradiographic technique (Daval *et al.*, unpublished data). The various compounds were injected at low doses in order to avoid marked peripheral effects. It was shown that CGS 21680 (0.01 mg/kg) substantially increased blood flow in almost all brain structures studied, whereas the A₁ agonist, 2-chloro-*N*⁶-cyclopentyladenosine (0.01 mg/kg), did not induce a significant effect.

5.3. Brain Energy Metabolism

Brain energy metabolism and blood flow are tightly coupled under both basal state and stimulation conditions, and evidence has accumulated that adenosine would constitute an important link between cerebral blood flow and tissue metabolism. In case of a mismatch between energy and/or oxygen demand and substrate delivery, adenosine concentrations in the interstitial fluid rise rapidly, resulting in adenosine-mediated adjustment of blood flow (Rubio *et al.*, 1975; Dirnagl *et al.*, 1994). In regard to this functional coupling, cerebral metabolic effects of adenosine and its analogues have not been explored extensively. Previous studies have shown that adenosine agonists either decrease local rates of energy metabolism (Grome and Stefanovich, 1986) or induce no significant changes (McBean *et al.*, 1989). More recently, we used selective A₁ and A₂ receptor agonists and antagonists to investigate receptor-specifically mediated cerebral metabolic effects of adenosine in rats by means of the quantitative autoradiographic method using 2-D-[¹⁴C]deoxyglucose (Nehlig *et al.*, 1994). The most prominent feature was that the A₂ agonist CGS 21680 (0.01 mg/kg, i.v.) induced a general depressant effect on local cerebral glucose utilization that was significant in 17 brain areas, such as cerebral cortex, hippocampal and white matter regions, as well as motor and limbic structures. The adenosine A₂ receptor antagonist 3,7-dimethyl-1-propargylxanthine (0.3 mg/kg) decreased glucose utilization in globus pallidus and increased it in the cochlear nucleus. By contrast, specific A₁ agonist and antagonist had very discrete influence on energy metabolism. The metabolic depressant effects of CGS 21680 were pronounced in globus pallidus, nucleus accumbens and caudate nucleus, these A_{2a}

receptor-enriched structures being known to be involved in the control of locomotor behavior. Although inherent to the technique itself that reveals energy metabolism changes throughout the nervous pathway affected by the drug tested, the mismatch between metabolic actions in extra-striatal areas and the A_{2a} receptor distribution might reflect, at least partly, activation of a widely distributed high-affinity binding sites for CGS 21680, as evoked above, as well as effects mediated through the low-affinity A_{2b} receptors in the brain. Similar observations have been made *in vitro*. Rat forebrain neurons in primary culture express functional adenosine receptors of both A₁ and A_{2a} subtypes (Nicolas *et al.*, 1994). At concentrations relevant for a specific interaction with A_{2a} receptors, CGS 21680 depressed the incorporation of 2-[³H]deoxyglucose into the cultured cells, whereas the specific antagonist 3,7-dimethyl-1-propargylxanthine had no significant consequences. Selective A₁ ligands also altered glucose metabolism, but additional experiments performed to analyze their effects on the hexose transport suggest that A₁ agonists and antagonists would rather act by competition at the glucose transporter (F. Nicolas and J.-L. Daval, unpublished data).

5.4. Respiratory System

Adenosine has been considered as a likely candidate in the modulation of central respiratory control in physiological conditions and a number of pathological situations. Also, the nucleoside has been implicated in the paradoxical ventilatory response to hypoxia in neonates. Barraco *et al.* (1990) have shown that cyclic AMP would serve as a second messenger in the central regulation of respiration that involves the solitary tractus nucleus, a major integrating site coordinating cardiopulmonary information and subsequent homeostatic responses. Microinjections of NECA into the caudal solitary tractus nucleus potently depressed respiration, concomitantly with marked increases in tidal volume. Such an action would be mediated through activation of adenosine receptors positively linked to adenylyl cyclase, presumably A_{2b} receptors. In contrast to the depressant central respiratory effects of adenosine, peripheral administration of the nucleoside stimulates respiration (Fuller *et al.*, 1987). The influence of various adenosine receptor ligands on ventilation have been evaluated in Rhesus monkeys in normal atmospheric conditions and during hypercapnia, hypoxia and hyperoxia (Howell, 1993). In normoxic conditions, NECA produced greater maximal stimulatory effects on respiratory frequency compared with CGS 21680. Adenosine A₂ receptor activation selectively increased the ventilatory response to hypoxia, but the A₂ agonists did not increase respiratory frequency during hyperoxia, and the author concluded that adenosine affects ventilation through peripheral, oxygen-sensitive mechanisms.

5.5. Gene Expression and Apoptosis

In response to a variety of cell stimuli, the immediate early gene c-fos encodes the nuclear Fos protein. In association

with Jun protein, Fos forms complexes that act as transcription factors at specific DNA sites, such as *API* (activator protein 1) and *CRE* (cyclic AMP responsive element) in the promoter region of target genes, and thus, influence their transcription. Little is known about the relationship between adenosine and immediate early gene regulation, and the interpretation of available data appears to be puzzling. In either fibroblasts or neuron-glia hybrid cells, it has been shown that treatment with adenosine receptor agonists, i.e., NECA and N⁶-cyclohexyladenosine, led to concentration-dependent expression of *c-fos* mRNA, a phenomenon inhibited by adenosine receptor antagonists (Gubits *et al.*, 1990). Since both cell lines tested are enriched in A₂ receptors and elevation of cyclic AMP levels is known to translate into *c-fos* expression in cultured cells, the above data would suggest that Fos induction may be a component of the A₂ receptor signal transduction. In a previous study performed in the mouse *in vivo*, Nakajima and co-workers (1989) demonstrated that i.p. administration of caffeine, an adenosine receptor antagonist, at both subconvulsive and convulsive doses, induced transient brain *c-fos* expression with a pattern of mRNA distribution that correlated with A_{2a} receptor striatal localization. In addition, caffeine-induced *c-fos* expression was blocked by the preliminary administration of NECA. Taken together, such data suggest that the caffeine action on *c-fos* may involve, at least partly, antagonistic properties at A_{2a} receptors. The adenosine A₂ receptor hypothesis was then challenged by Johansson *et al.* (1994), who reported that *c-fos*-labeled cells, analyzed by *in situ* hybridization in the rat striatum following caffeine administration, did not correspond exactly to A₂ receptor-positive neurons. These authors proposed that caffeine might induce *c-fos* by an increased release of excitatory amino acids secondarily to the blockade by the xanthine of A₁ receptors present on the terminals of corticostriatal projection neurons. Finally, in a very recent investigation, the induction of *c-fos* after administration of the A_{2a} receptor agonist CGS 21680 was analyzed in the striatum of normal rats and rats with unilateral 6-hydroxydopamine lesion of the dopaminergic nigrostriatal neurons (Morelli *et al.*, 1995). It was found that CGS 21680 (2.5 mg/kg) induced *c-fos* expression selectively in the lesioned striatum. The reversing effects of the D₂ agonist quinpirole or the muscarinic antagonist scopolamine prompted the authors to conclude that A_{2a} receptor activation could stimulate *c-fos* expression through an overproduction of cyclic AMP consecutively to dopamine denervation and/or by enhancement of acetylcholine-induced *c-fos* expression via muscarinic receptors.

At the intracellular level, adenosine was also reported to induce apoptosis in chick embryonic sympathetic neurons in primary culture (Wakade *et al.*, 1995). Addition of the nucleoside (1–100 μM) to the extracellular fluid inhibited neurite outgrowth and then killed sympathetic neurons supported by nerve growth factor, apoptotic cell death being revealed by DNA fragmentation. However, the authors

showed that adenosine receptors and the cyclic AMP signalling system were not implicated in neurotoxic actions of adenosine that rather would involve intracellular phosphorylation of the nucleoside and subsequent depletion of pyrimidine nucleotides.

5.6. Other Biological Effects

Both *in vivo* and *in vitro* studies have demonstrated that adenosine exhibits potent anti-inflammatory properties that are mediated through A₂ receptors (for a review, see Cronstein, 1994). Adenosine inhibits phagocytosis, generation of oxygen-derived free radicals and neutrophil adhesion, but does not affect degranulation or chemotaxis. Although activation of A₂ receptors is able to increase cyclic AMP levels in neutrophils, as observed following stimulation with chemoattractants, the A₂-mediated inhibition of leukocyte functions would not imply the participation of the cyclic AMP pathway, but rather might implicate the regulation of calcium fluxes. Nevertheless, it has been shown that the occupancy of adenosine A₂ receptors on neutrophils uncouples chemoattractant receptors from their stimulus-transduction proteins (Cronstein *et al.*, 1990).

Additionally, the adenosine A₂ receptor has been implicated in the renal functions, by decreasing glomerular filtration rate, stimulating renin release and increasing sodium excretion (Freissmuth *et al.*, 1987).

6. RECEPTOR REGULATION—PATHOPHYSIOLOGICAL RELEVANCE

6.1. Desensitization

Like their A₁ counterparts, A₂ receptors may be subject to desensitization by agonists (Hawkins *et al.*, 1988). It should be noted, however, that prolonged exposure of A_{2a} receptors to agonists did not lead to significant changes in receptor number or affinity in several reports (Ramkumar *et al.*, 1991; Chern *et al.*, 1993). Likewise, whereas an up-regulation of A₁ receptors has been demonstrated following chronic administration of caffeine, with synergistic enhanced behavioral effects of A₂ selective adenosine analogues, long-term exposure to adenosine antagonists, such as caffeine and theophylline, did not seem to affect either the density of A₂ receptors or their specific mRNA in the rodent striatum, although a tendency to an increased number of A₂ binding sites was noticed (Johansson *et al.*, 1993; Shi *et al.*, 1993). The different studies strongly suggest that treatments by selective agonists or antagonists might affect A₁ and A₂ receptors in a different way. In their recent study, Luthin *et al.* (1995) have reported that the use of [¹²⁵I]2-[2-(4-azido-3-iodophenyl)ethylamino]adenosine allows to quantify more accurately both high-affinity (G-protein-coupled) and low-affinity (G-protein-uncoupled) A_{2a} receptors in the rat brain, and the authors have proposed that radioiodinated selective ligands may be particularly relevant for investigating A_{2a} receptor-specific properties during desensitization.

6.2. Pathophysiological Implication

6.2.1. Psychiatric disorders. Besides pharmacologically induced changes, pathological alterations in A₂ receptors have been reported. A_{2a} receptor density is significantly decreased in the basal ganglia of Huntington's chorea patients, but unaltered in Parkinson's disease; such findings confirm the receptor localization on neurons that have their cell bodies in the striatum and are selectively destroyed in Huntington's disease (Martinez-Mir *et al.*, 1991).

Whereas adenosine-dopamine interactions provide a possible explanation for the central stimulatory effects of caffeine, at least at low doses (Fredholm *et al.*, 1993), the relationship between adenosine A₂ and dopamine D₂ receptors constitutes an opportunity for the treatment of basal ganglia disorders, as already pointed out by several authors. First, there is growing interest in the putative beneficial effects of A₂ receptor-related drugs in the treatment of Parkinson's disease and Huntington's chorea, two pathological states involving dopamine malfunctions. As extensively reviewed by Ferré *et al.* (1992), the well-known hypokinesia related to Parkinson's disease would be the consequence of an increased activity of the efferent striatal pathway arising from the GABAergic-enkephalin neurons, which contain D₂ and A₂ receptors, and the use of A₂ receptor antagonists, e.g., caffeine, would be expected to reduce the hypokinetic feature. Likewise, caffeine may be beneficial in advanced Huntington's chorea, whereas the activation of the A_{2a} receptors by selective agonists, provided they have low peripheral effects, might be of interest to stimulate the nervous pathway, and thus, ameliorate choreic movements in the early stages of disease. Also, due to their ability to reduce D₂ transduction, including that in the nucleus accumbens, A₂ receptor agonists might be useful as antipsychotic drugs, as illustrated by the demonstration of CGS 21680-induced catalepsy in rats (Ferré *et al.*, 1991a).

6.2.2. Hypoxia/ischemia. Changes in adenosine receptors also have been investigated by *in situ* hybridization and receptor autoradiography in the neonatal brain after hypoxia/ischemia induced by unilateral ligation of the common carotid artery followed by a transient period of hypoxia (Adén *et al.*, 1994). A clear-cut decrease in A_{2a} mRNA was found immediately after the hypoxic episode, especially in the caudate putamen, without significant alterations in the nucleus accumbens. These findings could also reflect impairment of the dopaminergic transmission under such conditions in the neonate, and the hypoxia/ischemia-induced down-regulation of adenosine functions probably enhances brain injury. In the adult animal, it has been reported that activation of A₂ receptors enhances ischemia-evoked excitotoxic amino acid release in the cerebral cortex, while 9-chloro-2-(2-furanyl)-5,6-dihydro-[1,2,4]-triazolo[1,5]quiazolin-5-imine monomethanesulfonate, a selective A_{2a} receptor antagonist, suppresses the ischemia-evoked release, and thus, would be potentially cerebroprotective (Simpson

et al., 1992; Gao and Phillis, 1994). On the other hand, accumulating data have demonstrated that activation of A₂ receptors by adenosine or selective agonists would limit brain damage following ischemia/reperfusion (for a review, see Rudolphi *et al.*, 1992). Indeed, although the A₁-mediated pre- and postsynaptic neuromodulation appears to be of special importance in the neuroprotective role of adenosine, A₂ effects would also contribute significantly, since activation of A₂ receptors located within the cerebral vasculature increases blood flow, oxygen and nutrient supply, decreases neutrophil adhesion to endothelial cells and subsequent capillary plugging, and exerts further anti-thrombotic effects by inhibition of platelet aggregation. By depressing the functions of stimulated neutrophils, A₂ receptors also reduce the generation of superoxide radicals and hydrogen peroxide, which are particularly deleterious to cell integrity. The latter action would be due to enhancement through A₂ receptors of the transient association of chemoattractant peptide-receptor complexes with the cytoskeleton (Cronstein *et al.*, 1990). Also, A₂ receptors could act at the level of microglial cells, a network of potential immunoeffector cells that are known to generate free radicals, and would increase glial glycogen breakdown, providing metabolizable substrate to the cells. The increase of adenosine levels in the cerebrospinal fluid during ischemia and reperfusion, coupled to parallel changes in pial arteriolar diameter, supports the role of A₂ receptors in the pathophysiology of brain reperfusion injury (Meno *et al.*, 1991). In addition, adenosine is of special importance in the fetal intrapartal adaptation (Irestedt *et al.*, 1989), as well as in the cerebral vasoadaptative changes to maintain autoregulation in newborns (Laudignon *et al.*, 1991), and several reports strongly suggest the potential usefulness of A₂-related compounds during the neonatal period.

6.2.3. Erythrocytosis. Erythrocytosis with associated increased production of erythropoietin is a well-known complication occurring after renal transplantation, and there is compelling evidence that erythropoietin production is modulated by adenosine. Such an effect would be mediated by A₂ receptors, since the mixed agonist NECA has been shown to increase erythropoietin production, an effect not observed with selective A₁ agonists. On the basis of these observations, Bakris and co-workers (1990) have evaluated the effects of theophylline in patients with erythrocytosis after renal transplantation, and they have shown that theophylline treatment resulted in a persistent attenuation of serum erythropoietin levels, concomitantly with a reduction of the hematocrit, leading to the avoidance of usually required phlebotomies. Such findings suggest that the use of A₂ selective antagonists may be of interest in this clinical situation.

6.2.4. Inflammatory Processes. Finally, as reported by Cronstein (1994), it should be mentioned that compounds acting selectively through adenosine A₂ receptors are excel-

lent candidates for the development of anti-inflammatory agents. Indeed, adenosine and its derivatives are potent inhibitors of inflammation in various animal models, and recent studies have established that the nucleoside modulates inflammation *in vivo* via A₂ receptors.

7. CONCLUSION

In light of recent biochemical, pharmacological and molecular studies, the knowledge of the structural and functional properties of A₂ receptors for adenosine has progressed considerably in the few past years, leading to the emergence of research investigations that focus more and more on the potential therapeutic applications of A₂-related compounds. Although numerous questions are still remaining and some data still require confirmation, the development of new agonist and antagonist ligands with high selectivity should finally allow to demonstrate in the near future that the A₂ receptors are certainly not the secondary adenosine receptors.

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CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE AND LEUCOCYTE CHEMOTAXIS *in vivo*

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- 1 The effect of local elevation of leucocyte cyclic adenosine 3',5'-monophosphate (cyclic AMP) content on the continued migration of leucocytes to a site of acute inflammation was studied in the pleural cavity of rats.
- 2 Leucocyte cyclic AMP levels were elevated by injecting with the irritant into the pleural cavity dibutyryl cyclic AMP alone or with theophylline.
- 3 The treatments both produced a marked reduction in leucocyte migration into a pleural reaction induced by immediate hypersensitivity, but had no effect on cell numbers in pleurisy induced either by pyrophosphate or urate crystals.

Introduction

The pleural cavity of rats has been shown to be a useful site for studying the effect of elevating endogenous leucocyte cyclic adenosine 3',5'-monophosphate (cyclic AMP) levels on the progression of acute inflammation *in vivo* (Deporter, Capasso & Willoughby, 1976; Deporter, 1977; Deporter, Dieppe, Glatt & Willoughby, 1977). The inflammatory reactions studied included an immediate hypersensitivity (Arthus) reaction and inflammation induced by calcium pyrophosphate crystals, both of which have relevance to human arthritis (Willoughby, 1976). In the present study the same two models have been used to study the effect of local elevation of leucocyte cyclic AMP concentration on the continued migration of leucocytes to a site of acute inflammation.

Methods

Male Wistar rats weighing 200-250 g were used. Pleurisy was induced by a mixture of pyrogen-free monoclinic and triclinic calcium pyrophosphate crystals as previously described (Deporter, Dieppe & Willoughby, 1976) or by immediate-hypersensitivity (reverse passive Arthus reaction) as described by Yamamoto, Dunn, Deporter, Capasso, Willoughby & Huskisson (1975). Briefly, bovine serum albumin was injected intravenously and 20-30 min later the animals were challenged intrapleurally with a purified rabbit antibody to bovine serum albumin. Pleurisy was also produced by injecting 1 ml of a solution of sodium biurate (10 mg/ml of saline) prepared according to the technique of Seegmiller, Howell & Malawista (1962).

To evaluate the effect of elevated leucocyte cyclic AMP content on the migration of more leucocytes into the pleural cavity in the three different pleural reactions, 2.45 mg dibutyryl cyclic AMP (Sigma) with and without 0.99 mg theophylline (BDH) was injected into the pleural cavity with the irritant. Both these drug treatments produce marked increases in the cyclic AMP content of leucocytes present in the pleural reactions under study (Deporter *et al.*, 1977; Deporter, 1977). Three hours after the onset of each reaction the animals were anaesthetized with ether and exanguinated via the carotid artery. Pleural exudates were withdrawn, blood-free, in siliconized Pasteur pipettes. After noting the volume of actual exudate in each animal, the pleural cavity was washed quickly with 2 ml of phosphate-buffered saline pH 7.4. Following brief agitation, the combined exudate and wash-out for each animal was sampled with a WBC pipette, diluted 1:20 with WBC diluent and counted within 30 minutes. Care was taken to count only samples free of WBC clumping within the WBC pipettes. WBC were counted 3 h after injection of the irritants, since by this time the three reactions studied were well advanced. Also, other experiments had shown that an effect on cyclic AMP levels in leucocytes by the drug treatments used could be maintained for 3 h but not longer (Deporter, unpublished results).

Results

The results are shown in Table 1. Dibutyryl cyclic AMP alone or in combination with the cyclic AMP

Table 1 Effect of local administration of dibutyryl cyclic AMP (2.45 mg) with or without theophylline (0.99 mg) on the migration of leucocytes into the pleural cavity of rats during three types of acute pleurisy

Type of pleurisy		Total WBC ($\times 10^6$)
Arthus-induced pleurisy	Control	60.4 \pm 3.7
	Dibutyryl cyclic AMP	39.2 \pm 6.0*
	Dibutyryl cyclic AMP and theophylline	36.4 \pm 8.4*
Pyrophosphate pleurisy	Control	33.8 \pm 5.2
	Dibutyryl cyclic AMP	34.0 \pm 5.1
	Dibutyryl cyclic AMP and theophylline	36.5 \pm 7.1
Urate pleurisy	Control	28.2 \pm 3.7
	Dibutyryl cyclic AMP	30.8 \pm 3.1
	Dibutyryl cyclic AMP and theophylline	26.3 \pm 4.9

* $P < 0.05$.

phosphodiesterase inhibitor theophylline significantly decreased leucocyte counts in intrapleural Arthus reactions. In contrast, in pleurisy produced by pyrophosphate or urate crystals, (Table 1) neither drug treatment produced a significant change in the numbers of migrating leucocytes. Moreover, neither drug treatment produced significant changes in exudate volume over control values in any of the three models studied (results not shown).

Discussion

The mechanism by which a local elevation of leucocyte cyclic AMP content could reduce further leucocyte migration to a site of inflammation induced by immediate hypersensitivity is not known. However, since polymorphonuclear leucocytes (PMNs) engaged in phagocytosis of a variety of particles including antigen-antibody complexes release chemotactic factors for other PMNs (Tse & Phelps, 1970; Keller & Borel, 1971; Henson, 1972), it is conceivable that the elevated cyclic AMP levels inhibited the release of chemotactic factors into the Arthus-induced pleural effusions. The different effect of elevated cyclic AMP on cell migration in the different types of acute inflammation studied is difficult to explain. It is not due to an inhibition of particle ingestion since other experiments using the same models have shown that dibutyryl cyclic AMP and theophylline have no effect on leucocyte phagocytosis *in vivo* (Deporter *et al.*, 1977). However, the difference may be related to the fact that only the pleurisy induced by immediate hypersensitivity is complement-dependent (Yamamoto *et al.*, 1975). The two types of crystal-induced inflammation are not complement-dependent (Willoughby, Dunn, Yamamoto, Capasso, Deporter & Giroud, 1975;

McCarty & Kozin, 1975). Complement generates factors that are chemotactic for PMNs (Ward, 1974) but the effect of cyclic AMP on the generation of these factors is not yet known and requires *in vitro* investigation.

The present results with urate crystals are in disagreement with those of Tse & Andrews (1973) who showed that the release of a chemotactic factor from PMNs following the intra-articular injection of urate crystals in dogs could be inhibited by injecting cyclic AMP with the crystals. The conflicting results could be due to differences in crystal preparation if, for example, Tse & Andrews did not include heat sterilization at 180°C. Non-heated urate crystals are known to activate the complement system (McCarty & Kozin, 1975). Also, since cyclic AMP levels in the dog leucocytes were not monitored, the inhibition of chemotaxis observed may not have been associated with elevated leucocyte cyclic AMP content. Cyclic AMP penetrates cell membranes less effectively than its dibutyryl derivative and is rapidly degraded by cyclic AMP phosphodiesterase (Robison, Butcher & Sutherland, 1971). Furthermore, *in vitro* butyrylated cyclic AMP derivatives and theophylline but not exogenous cyclic AMP mimic the effects of increased intracellular cyclic AMP (Drezner, Neelson & Lebovitz, 1976).

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CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE AND THE MECHANISM OF ACTION OF THREE COMMON ANTI-INFLAMMATORY DRUGS

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- 1 The effects of indomethacin, dexamethasone and colchicine on cyclic adenosine 3',5'-monophosphate (cyclic AMP) concentration in leucocytes during a crystal-induced pleurisy in rats were studied.
- 2 Each of the drugs significantly increased leucocyte cyclic AMP content within 3 h of the injection of crystals.
- 3 By 6 h, leucocyte cyclic AMP levels were returning toward control levels and could not be sustained at the higher level by an additional administration of the respective anti-inflammatory drug.

Introduction

Recent experiments have established that cyclic adenosine 3',5'-monophosphate (cyclic AMP) plays a central role in acute inflammation (Willoughby, Dunn, Yamamoto, Capasso, Deporter & Giroud, 1975; Yamamoto, Dunn, Capasso, Deporter & Willoughby, 1975a; Yamamoto, Dunn, Deporter, Capasso & Willoughby, 1975b; Deporter, Dieppe & Willoughby, 1976a). A common time course pattern for leucocyte cyclic AMP levels was demonstrated in different types of inflammation in the pleural cavities of rats and guinea-pigs. Following an initial and variable rise 1 h after intrapleural injection of an irritant, the concentration of cyclic AMP in pleural exudate leucocytes always fell precipitously below basal levels by 3 h and returned towards basal levels only as the inflammatory reactions subsided. These results suggested that an inflammatory reaction will proceed normally only when the concentration of cyclic AMP in participating leucocytes is lower than in resting leucocytes, and that artificial elevation of cyclic AMP levels in leucocytes might have an anti-inflammatory effect. The effect of the anti-inflammatory drugs, indomethacin, dexamethasone and colchicine on the content of cyclic AMP in inflammatory leucocytes was therefore investigated. Pleurisy induced in rats by calcium pyrophosphate crystals was used. This model has proved useful in the evaluation of drugs for the treatment of human arthritis (Willoughby, 1976).

Methods

Pyrophosphate crystal pleurisy was produced in male Wistar rats (200 to 250 g) as previously described (Deporter *et al.*, 1976b). At 3 h and 6 h after the injection of crystals, the cyclic AMP content of

pleural exudate leucocytes was determined by protein-binding radioassay (Deporter, Dieppe, Glatt & Willoughby, 1977).

Indomethacin was administered orally in 0.9% w/v NaCl solution (saline) at a dose of 3 mg/kg body wt. 12 h and 1 h before intrapleural injection of pyrophosphate crystals. Dexamethasone was given orally at 2 mg/kg in saline 1 h before the reaction. Colchicine was injected into the tail vein at a dose of 0.2 mg/kg body wt. 1 h before the intrapleural reaction was elicited.

In one set of experiments the animals were given an additional dose of their respective drugs 3 h after the onset of the reaction, and the content of leucocyte cyclic AMP was determined at 6 h.

Results

The effects of the three drugs studied on leucocyte cyclic AMP levels at 3 and 6 h after intrapleural injection of pyrophosphate crystals are shown in Figure 1. While indomethacin produced a small but significant increase in leucocyte cyclic AMP content, dexamethasone and colchicine increased the content dramatically. By 6 h the effect of indomethacin had completely worn off but dexamethasone and colchicine still maintained a significant, albeit smaller, increase over control levels of cyclic AMP. However, an additional dose of the respective drug given 3 h after the injection of crystals had no additional effect on leucocyte cyclic AMP levels at 6 h (Figure 2).

Discussion

It is not certain how indomethacin, dexamethasone and colchicine might affect leucocyte cyclic AMP con-

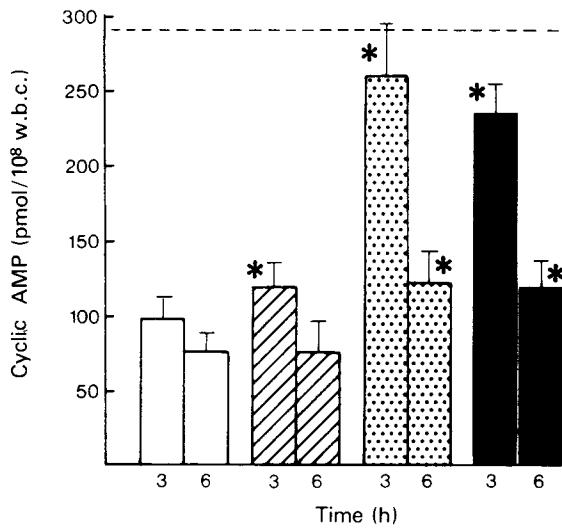


Figure 1 The effects of indomethacin (hatched columns), dexamethasone (stippled column) and colchicine (solid columns) on cyclic adenosine 3',5'-monophosphate (cyclic AMP) content of leucocytes (w.b.c.) from 3 and 6 h pyrophosphate-induced pleural exudates. * Significant difference ($P < 0.05$) from values in control exudates (open columns); vertical lines show s.e. mean. The broken line denotes the concentration of cyclic AMP in 'resting' leucocytes obtained from the pleural cavity of animals not injected with crystals or drugs (s.e. mean \pm 45 pmol).

tent. Studies on chicken epiphyseal and articular cartilage (Newcombe, Thanassi & Ciosek, 1974), human synoviocytes (Ciosek, Ortell, Thanassi & Newcombe, 1974), toad bladder (Flores & Sharp, 1972), frog skin (Hall & O'Reagan, 1975) and purified beef heart phosphodiesterase (Stefanovich, 1974) have shown that indomethacin inhibits not only prostaglandin synthesis but also cyclic AMP phosphodiesterase activity and thereby raises cellular cyclic AMP content. Dexamethasone and other corticosteroids inhibit phosphodiesterase activities and increase cellular cyclic AMP in *in vitro* preparations such as rat hepatoma cells (Manganiello & Vaughan, 1972), rat testis (Schmidtke, Wienker, Flugel & Engel, 1976) and purified beef heart phosphodiesterase (Stefanovich, 1974).

The effect of colchicine on cyclic AMP phosphodiesterase activity is not known. Colchicine increases leucocyte cyclic AMP content *in vitro* and enhances the increase produced by adenylate cyclase stimulators such as isoprenaline and prostaglandin E₁ (Rudolph, Greengard & Malawista, 1977). However, colchicine may produce these effects by stimulating prostaglandin synthesis by leucocytes (Glatt, Graf &

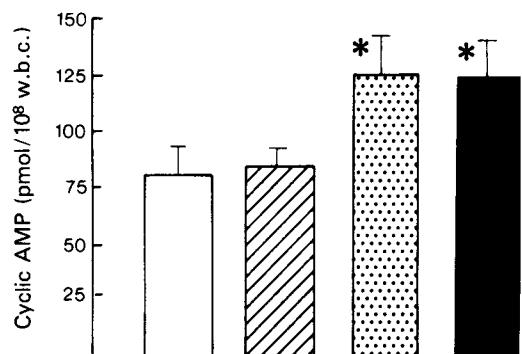


Figure 2 The effects of an additional dose of indomethacin (hatched column), dexamethasone (stippled column) or colchicine (solid column) on cyclic adenosine 3',5'-monophosphate (cyclic AMP) content of leucocytes from 6 h exudates. * Significant difference ($P < 0.05$) from values in control exudates (open column). Vertical lines show s.e. mean.

Brune, 1975). This would also help to explain why higher levels of cyclic AMP occur in animals treated with colchicine than in those treated with indomethacin where prostaglandin synthesis is inhibited.

We suggest that one or more of the anti-inflammatory effects of indomethacin, dexamethasone and colchicine is mediated through an elevation of leucocyte cyclic AMP content. This rise could then affect the inflammatory reaction by inhibiting the release of inflammatory mediators such as histamine. This hypothesis is corroborated by the finding of Thomas & Whittle (1976) that indomethacin inhibited histamine release from isolated challenged mast cells of the rat, probably through inhibition of mast cell phosphodiesterase activity. A similar inhibition of histamine release *in vitro* (Orange, Kaliner, Larama & Austen, 1971) and *in vivo* (Deporter, Capasso & Willoughby, 1976a) can be produced by elevation of leucocyte cyclic AMP levels with dibutyryl cyclic AMP and another phosphodiesterase inhibitor, theophylline.

Long-term alteration of leucocyte cyclic AMP levels by anti-inflammatory drugs may not be feasible since at 6 h the effect of indomethacin on cyclic AMP content was negligible, and of dexamethasone and colchicine greatly reduced. A second dose of the drugs did not affect the leucocyte cyclic AMP content. The apparent refractoriness could be due to a feedback regulator for cyclic AMP phosphodiesterase, similar to the one postulated to regulate adenylate cyclase activity (Ho & Sutherland, 1975; Ho, Russell & Asakawa, 1975).

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Anti-inflammatory activities of cAMP-elevating agents: enhancement of IL-10 synthesis and concurrent suppression of TNF production

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Abstract Interleukin-10 (IL-10) and tumor necrosis factor (TNF) exert key roles in some acute and chronic inflammatory diseases. In this study we investigated (1) the potency of different cAMP-elevating agents in enhancing IL-10 synthesis, (2) the involvement of protein kinase A in this enhancement, and (3) the mutual dependence of cAMP-enhanced IL-10 formation and cAMP-suppressed TNF synthesis. Rolipram, a specific phosphodiesterase inhibitor and cicaprost, a prostacyclin analogue, were applied as cAMP-elevating agents. The stable cAMP antagonist (Rp)-cAMPS was used to abrogate activation of protein kinase A. Human peripheral blood mononuclear cells were stimulated with lipopolysaccharide (LPS). TNF was quantified by radioimmunoassay, IL-10 by enzyme-linked immunosorbent assay, and mRNA by reverse transcriptase-polymerase chain reaction. After LPS stimulation alone 253 ± 45 pg/mL IL-10 was synthesized, which increased to 644 ± 117 pg/mL in the presence of 1 μ M rolipram. (Rp)-cAMPS reversed this increase of IL-10 formation. In the same samples, the LPS-stimulated production of TNF was markedly attenuated by rolipram or cicaprost. A kinetic analysis revealed a significant increase in TNF production before IL-10 formation was detectable. These results demonstrate that (1) cAMP-elevating agents enhance IL-10 synthesis and suppress TNF production; (2) these regulatory functions of cAMP-elevating agents are mediated by activation of protein kinases A; (3) suppression of TNF synthesis by cAMP in the early phase is not mediated by endogenous IL-10. Taken together, rolipram and cicaprost exert a dual regulatory function by enhancing IL-10 formation and attenuating TNF synthesis. *J. Leukoc. Biol.* 63: 101–107; 1998.

Key Words: human mononuclear cells · phosphodiesterase inhibitor · rolipram · cicaprost

INTRODUCTION

Tumor necrosis factor (TNF) has been identified as one of the earliest secretory products, released as soon as 90 min after

stimulation of monocytes. As such, TNF orchestrates several pro-inflammatory processes leading to acute and chronic inflammatory diseases. Evidence for this central role of TNF derives from cell culture experiments and, more recently, from successful anti-TNF antibody treatment of rheumatoid arthritis [1], inflammatory bowel disease [2, 3], and Jarisch-Herxheimer reaction [4] in humans. Regulatory mechanisms leading to diminished TNF production are therefore of immediate clinical interest. Several endogenous mediators have been described that attenuate TNF synthesis, including adenosine [5, 6], cAMP [7, 8], nitric oxide [9, 10], interleukin-4 (IL-4) [11], transforming growth factor- β [12], and IL-10 [13]. This study focuses on cAMP-mediated enhancement of IL-10 synthesis and suppression of TNF synthesis.

IL-10 has initially been identified as a T_{H2} cell product inhibiting T_{H1} cell proliferation, development, and function [14]. IL-10 is synthesized by various other cells, such as activated B cells and B cell lymphoma cells [15, 16], monocytes and macrophages, keratinocytes [17], and mast cells [18]. According to its primary name, cytokine synthesis inhibitory factor, IL-10, inhibits the production of several pro-inflammatory cytokines including TNF and IL-1 β in monocytes and macrophages [18]. This activity of endogenous IL-10 limits the inflammatory response. A protective effect of IL-10 has been demonstrated in a murine model of lethal endotoxemia induced by intraperitoneal injection of endotoxin [19]. Currently, phase III trials to investigate the efficacy of intravenous IL-10 administration in humans with inflammatory bowel disease are under way [20]. Alternatively to the administration of IL-10 itself, experimental therapeutics to enhance endogenous induction of IL-10 are being investigated. Among these, cAMP-elevating agents appear promising because it is known that cAMP elevation mediates TNF suppression.

Various cAMP-elevating agents such as rolipram and cicaprost have been demonstrated to inhibit TNF synthesis [21, 22]. We recently showed that the combination of the endogenous TNF suppressor IL-10 with the pharmacological agents rolipram or

Abbreviations: IL-10, interleukin-10; TNF, tumor necrosis factor; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction.

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cicaprost led to an enhanced suppression of lipopolysaccharide (LPS)-induced TNF production [23]. Although the suppressive effect of cAMP-elevating agents on TNF synthesis has been investigated by numerous reports, beginning in 1986 [24], only a few studies have focused on the effect of phosphodiesterase inhibitors on the synthesis of IL-10 [25, 26].

In this study the enhancement of IL-10 synthesis and the concurrent suppression of TNF production by cAMP-elevating agents was investigated at the mRNA and at the protein level in human mononuclear cells. Furthermore, the signal transduction engaged by the cAMP-elevating agents was examined. Finally, the mutual dependence of IL-10 and TNF concerning their regulation by cAMP was investigated.

MATERIALS AND METHODS

Preparation of mononuclear cells

Heparinized blood was drawn from healthy fasting volunteers who had been without medication for at least 2 weeks. The peripheral blood mononuclear cell (PBMC) fraction was obtained by gradient centrifugation over Ficoll-Hypaque (Biochrom, Berlin) as described previously [7, 27]. As a modification of the protocol, isolation was performed in tubes containing a horizontal porous filter disc over the Ficoll layer (Leucosep® tubes, Greiner, Frickenhausen, Germany) to facilitate layering of blood. RPMI culture medium was supplemented with 2 mM L-glutamine, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Sigma, München, Germany). Cells were suspended at 5.0×10^6 cells/mL in this medium supplemented with 2% heat-inactivated sterile human serum (from a donor with AB blood group).

Preparation of the compounds

Rolipram (racemate of 4-[3'-cyclopentyloxy-4'-methoxyphenyl]-2-pyridone, from Schering AG, Berlin, Germany), supplied in powder form, was dissolved in RPMI medium by vigorous vortexing. Cicaprost (Schering AG) was directly diluted in RPMI-1640 medium containing 2% sterile heat-inactivated human serum. IL-10 (Boehringer Mannheim, Germany) dissolved in RPMI medium, supplemented with 5% sterile heat-inactivated human serum, was diluted in RPMI medium containing 2% human serum. The inhibitors rolipram or cicaprost (62.5 µL) and IL-10 (62.5 µL) were pipetted into wells of a 48-well culture plate (Falcon® Becton Dickinson, Lincoln Park, NJ). Rp-adenosine-3',5'-cyclic phosphorothioate [(Rp)-cAMPS, BIOLOG Life Science Institute, Bremen, Germany], a diastereomer of adenosine-3',5'-cyclic phosphorothioate, is known to inhibit competitively the cAMP-induced activation of protein kinase A. Sp-adenosine-3',5'-cyclic phosphorothioate [(Sp)-cAMPS, BIOLOG Life Science Institute] is an activator of protein kinase A (type I and II). (Rp)-cAMPS and (Sp)-cAMPS, supplied in powder form, were dissolved in the supplemented RPMI-1640 medium. In some experiments, cells were preincubated for 20 min with (Rp)-cAMPS before LPS stimulation and addition of the cAMP-elevating agents' cells. To exclude contaminations with endotoxin all reagents used were tested in the *Limulus* amebocyte lysate assay according to the manufacturer's protocol (Chromogenix, Charlston, SC) and were found to be endotoxin negative (endotoxin content less than 6.0 pg/mL).

Cell stimulation

For induction of cytokines lipopolysaccharide (LPS; from *Escherichia coli* 055:B5; Sigma) was freshly diluted from a frozen aliquot with the supplemented RPMI medium containing 2% endotoxin-free human albumin (Behringwerke, Marburg, Germany). LPS, 125 µL, was added into wells of the 48-well culture plate containing 125 µL of diluted compounds as described above. Subsequent addition of 250 µL PBMC suspension with a concentration of 5.0×10^6 cells/mL gave a final volume of 500 µL and a final LPS concentration of 10 ng/mL. The incubation period (ranging from 1 to 20 h) at 37°C in 5% CO₂ and 90%

humidified air was terminated by freezing plates at -70°C to obtain combined lysate plus supernatant.

Measurement of TNF and IL-10

TNF was determined by specific radioimmunoassay as described [28]. To rationalize sample processing, a 96-microtube plate system with single polypropylene tubes (Sarstedt, Nümbrecht, Germany) was used. The sample (50 µL) was added to 50 µL of diluted polyclonal anti-TNF rabbit antiserum and 50 µL 1% rabbit IgG and was incubated overnight. Bolton Hunter-labeled ¹²⁵I-TNF (50 µL; Du Pont-New England Nuclear, Bad Homburg, Germany) was added on the second day. After another overnight incubation, 250 µL of second antibody (sheep anti-rabbit IgG) in 6% polyethylene glycol was added. TNF concentrations were calculated from a standard curve of human recombinant TNF (supplied by the National Institute for Biological Standards and Control, Potters Bar, UK) ranging from 0.02 to 10 ng/mL. IL-10 were determined after one freeze-thaw cycle by a commercial IL-10 ultrasensitive enzyme-linked immunosorbent assay (Bio-Source, International Inc.).

Cell stimulation and preparation of RNA

For detection of TNF and IL-10 mRNA by polymerase chain reaction, 500 µL LPS was added into wells of the six-well culture plate containing 500 µL of diluted cicaprost or rolipram. Addition of 1 mL PBMC suspension with a concentration of 5.0×10^6 cells/mL gave a final volume of 2 mL. The cells were incubated for 4 h. Cells were harvested by centrifugation at 2000 g for 5 min, culture medium was removed before lysing the cells with 4 M guanidine isothiocyanate containing β-mercaptoethanol (0.1 mM). Total RNA was extracted by phenol-chloroform-isoamylalcohol, with a second precipitation step.

cDNA synthesis and oligonucleotides

Aliquots of each total RNA extraction were reverse-transcribed simultaneously into cDNA with the use of M-MLV reverse-transcriptase according to the manufacturer's protocol (GIBCO-BRL, Eggenstein, Germany). One microliter of the resulting cDNA was then used for amplification with PCR (see below). Oligonucleotide primers specific for human β-actin, TNF, and IL-10 mRNA were synthesized according to known cDNA sequences [29–31] (Mikrogen, München, Germany). Nested internal primers were chosen in close proximity to the external primers at a distance of two or three single bases, resulting in diagnostic segments of 239 base pairs (bp; β-actin), 192 bp (TNF), and 280 bp (IL-10). Final concentrations of primers ranged from 0.01 to 6 µM.

cDNA amplification

Polymerase chain reaction (PCR) and mRNA quantification was performed as described by Kaminski et al. [32].

Statistical analysis

Results are given as means ± SEM. The paired two-tailed Student's *t*-test was performed for comparisons of means. Differences were considered statistically significant for *P* < 0.050. All statistical analyses were performed using Stat-View 512 software (Abacus Concepts, Calabasas, CA).

RESULTS

Enhancement of LPS-induced IL-10 synthesis and concurrent suppression of TNF formation by cAMP-elevating agents

LPS (10 ng/mL) stimulation of PBMC for 20 h induced IL-10 synthesis of 313 ± 24 pg/mL (Fig. 1A) and TNF synthesis of 4.5 ± 1.2 ng/mL (Fig. 1B). After stimulation in the presence of the specific type IV PDE inhibitor rolipram (100 nM), an increase of IL-10 synthesis to 425 ± 16 pg/mL (*P* = 0.016) could be detected with a parallel suppression of TNF synthesis to 2.5 ± 1.0 ng/mL (*P* = 0.010). A plateau of IL-10 formation was

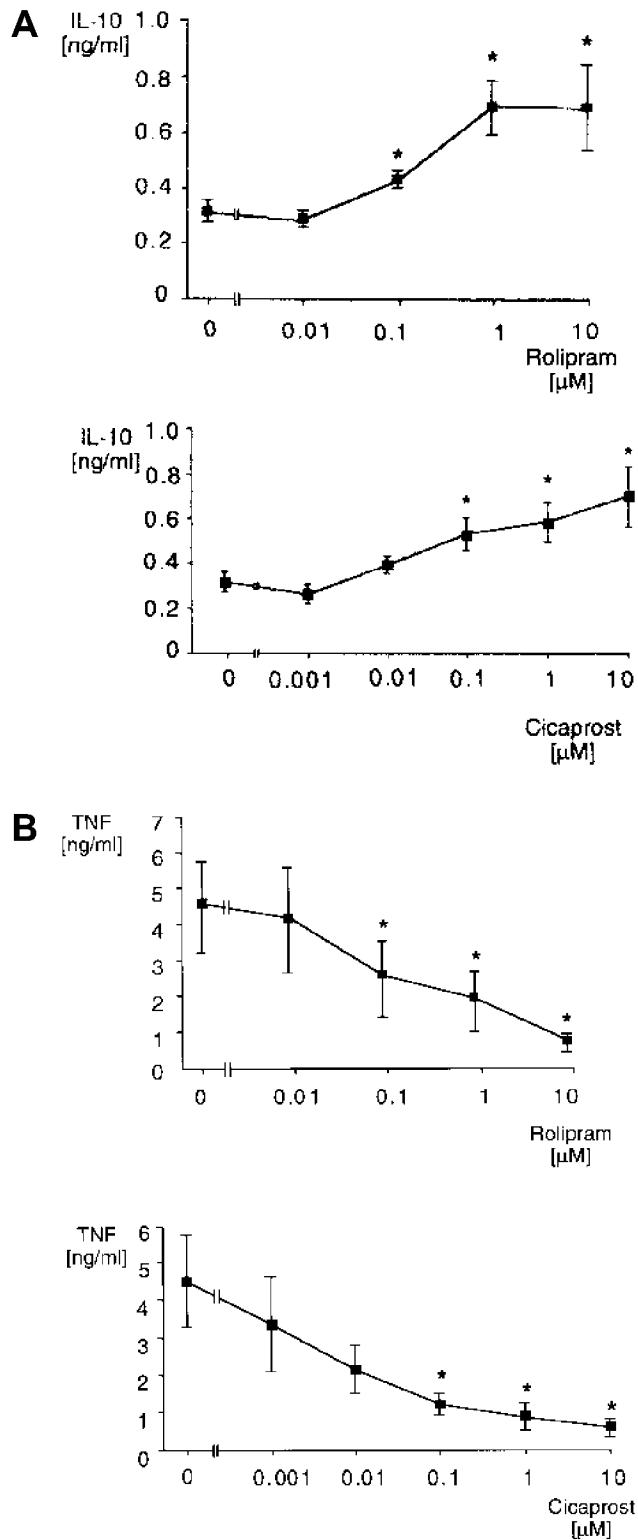


Fig. 1. Dose-dependent enhancement of LPS-induced IL-10 synthesis by rolipram and cicaprost (A) and parallel inhibition of LPS-induced TNF synthesis (B). Human PBMC were stimulated by 10 ng/mL LPS for 20 h in the presence of increasing concentrations of the cAMP-elevating agents. Total, i.e. cell-associated and secreted IL-10 and TNF were quantified by ELISA and RIA, respectively. Bars represent means \pm SEM of $n = 4$ different donors. *Statistically significant difference at $P < 0.05$.

reached at a concentration of 1 μ M rolipram (689 ± 147 pg/mL IL-10) paralleled by a marked inhibition of TNF synthesis (1.9 ± 0.8 ng/mL). Stimulation in the presence of the stable prostacyclin analogue cicaprost (100 nM) led to an increase of IL-10 synthesis to 528 ± 48 pg/mL ($P = 0.050$). At this concentration TNF was suppressed to 2.1 ± 0.5 ng/mL TNF ($P = 0.005$). Maximal concentration of IL-10 (700 ± 135 pg/mL IL-10) was achieved at 10 μ M cicaprost with a suppression of TNF synthesis to 0.6 ± 0.2 ng/mL. In control experiments, i.e. cells incubated with either of the cAMP-elevating agents but without LPS, the IL-10 concentrations were less than 20 pg/mL and TNF concentrations were below the detection limit of 20 pg/mL (not shown in Fig. 1).

Effect of cAMP-elevating agents on LPS-induced IL-10 mRNA

RT/PCR analysis of IL-10 mRNA was performed to elucidate whether the enhancement observed in protein synthesis occurs on a pretranslational level (Fig. 2A). PBMC incubated without LPS for 4 h contained little IL-10 mRNA (0.30 of standardized IL-10 mRNA). LPS (10 ng/mL) increased the amount of mRNA to 0.43 of standardized IL-10 mRNA. Each of the cAMP-elevating agents significantly enhanced IL-10 mRNA: rolipram (1 μ M) to 0.89 of standardized IL-10 mRNA and cicaprost to 1.00 of standardized IL-10 mRNA. In the identical samples TNF mRNA was quantified (Fig. 2B). Cells incubated without LPS contained only a little TNF mRNA. This was strongly induced by LPS. Rolipram and cicaprost significantly decreased TNF mRNA in comparison to LPS stimulation alone.

Reversal of enhancement of IL-10 synthesis by the specific protein kinase A inhibitor (Rp)-cAMPs

LPS-stimulated PBMC were stimulated in the presence of a cAMP-elevating agent alone or in combination with 300 μ M or 1 mM (Rp)-cAMPs (Fig. 3). Cells incubated with LPS alone synthesized 253 ± 45 pg/mL IL-10. After incubation with 1 μ M rolipram IL-10 synthesis was elevated to 645 ± 118 pg/mL ($P = 0.043$; means of $n = 4$ different donors \pm SEM). Simultaneous addition of 300 μ M (1 mM) (Rp)-cAMPs decreased IL-10 synthesis to 497 ± 44 pg/mL (319 ± 55 pg/mL; $P = 0.038$). In a similar pattern 1 mM (Rp)-cAMPs reversed cicaprost-enhanced IL-10 synthesis from 735 ± 140 pg/mL to 427 ± 66 pg/mL ($P = 0.050$). This indicates that for either agent the activation of protein kinases A is a necessary intermediate step for enhancement of LPS-induced IL-10 synthesis.

Dose-dependent induction of IL-10 synthesis by the cAMP agonist (Sp)-cAMPs

Finally, we examined whether increased availability of cAMP is also a sufficient signal to enhance LPS-induced IL-10 synthesis. (Sp)-cAMPs is a stable, cell permeable cAMP analogue that activates protein kinase A (type I and II) [33]. PBMC were stimulated by 10 ng/mL LPS in the presence of different concentrations of (Sp)-cAMPs (Fig. 4). Cells incubated with LPS alone synthesized 212 pg/mL IL-10. The addition of 100 μ M (Sp)-cAMPs markedly enhanced IL-10 formation to 604 pg/mL. Maximal IL-10 production of 926 pg/mL occurred in the presence of 500 μ M (Sp)-cAMPs. (Sp)-cAMPs (1 mM) alone induced a small increase in IL-10 formation to 60 pg/mL.

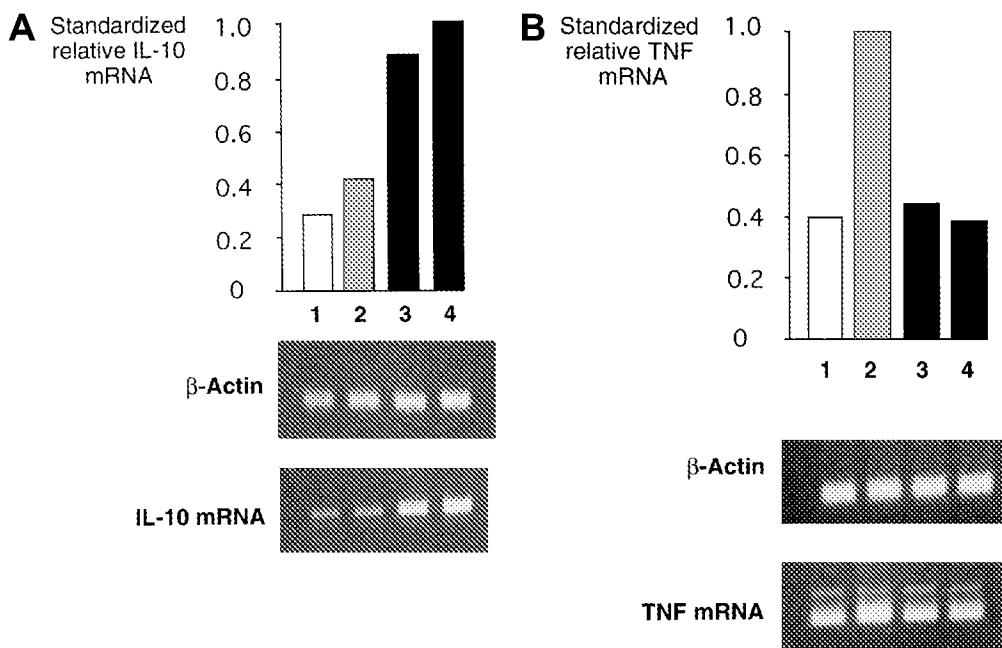


Fig. 2 Increase of IL-10 mRNA by rolipram and cicaprost. Four hours after stimulation of PBMC with LPS total RNA was harvested and RT-PCR analysis was performed. The conditions of the four lanes in both panels (A, IL-10 mRNA; B, TNF mRNA) are (1) cells without stimulus, (2) cells stimulated with LPS 10 ng/mL, (3) cells stimulated with LPS in the presence of 1 μ M rolipram, (4) cells stimulated with LPS in the presence of 1 μ M cicaprost.

Kinetics of IL-10 and TNF synthesis induced by LPS in the presence of rolipram

PBMC were incubated with 10 ng/mL LPS in the presence or absence of 1 μ M rolipram for different time intervals (Fig. 5).

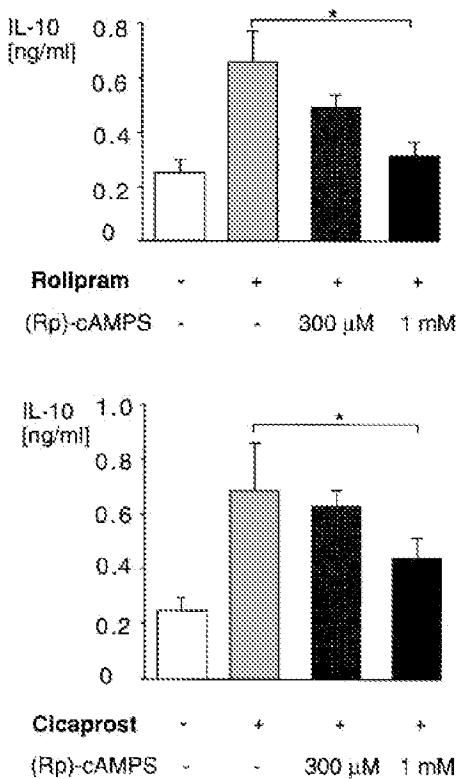


Fig. 3 (Rp)-cAMPS reverses rolipram- and cicaprost-enhanced IL-10 production. PBMC were stimulated by 10 ng/mL LPS in the presence of cicaprost (100 nM) or rolipram (1 μ M) alone or in combination with (Rp)-cAMPS (300 μ M or 1 mM). Bars represent means \pm SEM of $n = 4$ different donors. *Statistically significant decrease of IL-10 synthesis at $P < 0.050$.

With LPS alone IL-10 accumulation could be detected starting at 6 h after stimulation (79 ± 29 pg/mL). When LPS stimulation was performed in the presence of 1 μ M rolipram an increase of IL-10 synthesis could be observed starting at 4 h (50 ± 10 pg/mL; means of $n = 4$ different donors \pm SEM). Within the time frame studied maximal IL-10 production was reached at 20 h. At that time incubation in the presence of rolipram induced IL-10 synthesis of 695 ± 63 pg/mL compared to 311 ± 37 pg/mL IL-10 after LPS stimulation alone. Thus, rolipram significantly enhances IL-10 synthesis beginning at 4 h after LPS stimulation.

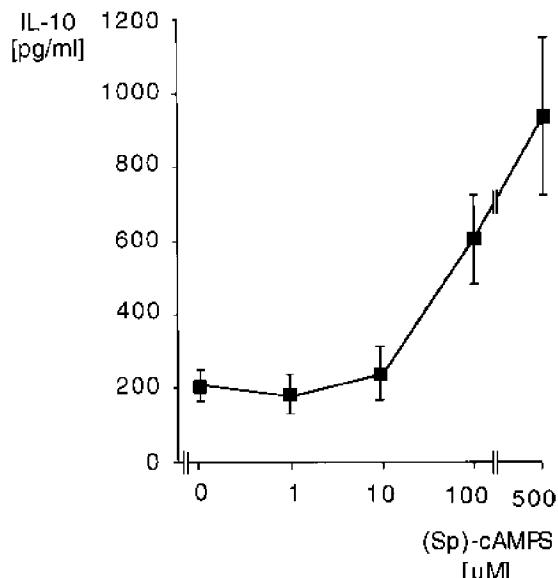


Fig. 4 Dose-dependent enhancement of IL-10 synthesis by the cAMP agonist (Sp)-cAMPS. PBMC were stimulated with 10 ng/mL LPS in the presence of different concentrations of (Sp)-cAMPS. IL-10 synthesis was increased to 604 pg/mL ($P = 0.024$) by 100 μ M (Sp)-cAMPS. Maximal IL-10 synthesis (926 pg/mL) occurred at 500 μ M (Sp)-cAMPS. Bars represent means \pm SEM of $n = 4$ different donors.

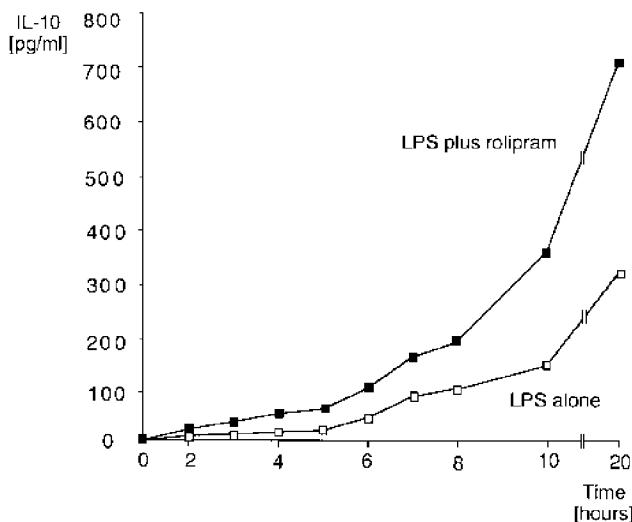


Fig. 5. Kinetic analysis of IL-10 production. PBMC were stimulated by 10 ng/mL LPS in the presence or absence of rolipram (1 μ M). At time points indicated total, i.e., cell-associated and secreted IL-10 was quantified by ELISA. Results are shown as means of $n = 4$ different donors.

This enhancement is approximately twofold at all time points studied. In the same samples we could demonstrate an inhibition of LPS-induced TNF synthesis by rolipram beginning at 90 min, the earliest time point of TNF detection (data not shown).

DISCUSSION

In this study we demonstrate marked enhancement of LPS-stimulated IL-10 synthesis (transcript and protein) by rolipram and cicaprost in human PBMC. Concurrently, a strong inhibition in TNF formation could be confirmed. The mediating signal transduction pathway engages protein kinases A as determined by the reversal of both effects in the presence of the cAMP antagonist (Rp)-cAMPS. Finally, the time pattern of IL-10 production and TNF formation suggests that cAMP-mediated TNF suppression, at least during the initial phase of 4 h, cannot be ascribed to endogenous IL-10.

The anti-inflammatory functions of cAMP-elevating agents have been demonstrated in several cell systems such as the inhibition of neutrophil activation, of leukocyte adherence to endothelial cells, and of monocyte TNF synthesis [7, 34]. Concerning TNF, most cAMP-elevating agents employed so far had to be applied at higher concentrations to reach significant suppression (*in vitro*) than could be reached by systemic administration *in vivo*. It therefore appeared promising when the specific type IV phosphodiesterase inhibitor rolipram was identified as an 800-fold more potent TNF suppressor (on a molar basis) than the unspecific phosphodiesterase inhibitor pentoxifylline [22]. In 1995 two studies demonstrated that the production of the anti-inflammatory cytokine IL-10 is markedly enhanced by cAMP-elevating agents [25, 26]: Platzer et al. applied iloprost as an activator of adenylyl cyclase in human PBMC, whereas Kambayashi et al. used rolipram in thioglycollate-elicited murine macrophages. This study is therefore the first to demonstrate enhanced IL-10 formation by rolipram in cells of human origin.

The pathway leading to IL-10 enhancement by cAMP-

elevating agents has not yet been elucidated. We used the specific cAMP antagonist (Rp)-cAMPS, the only known effective inhibitor of the type I and II protein kinase A, to investigate whether protein kinase A activation is a necessary intermediate in cAMP-enhanced IL-10 synthesis. (Rp)-cAMPS has recently been shown to inhibit activation of protein kinase A by blocking the cAMP-induced conformational transition [33]. The two regulatory subunits and the two catalytic subunits of the PKA are locked by (Rp)-cAMPS preventing the catalytic subunits from dissociating. The addition of (Rp)-cAMPS in our experiments reversed rolipram- and cicaprost-enhanced IL-10 production. This finding indicates that activation of protein kinase A is a necessary step in IL-10 enhancement by these two agents.

Concentrations of 1 mM (Rp)-cAMPS appear high when compared to physiological concentrations of cAMP. But several facts contribute to the need for high concentrations to see a specific effect: cell permeability, compartmentalization of cAMP, and finally, kinetics of cAMP formation. The determination of two cytokines regulated in diverse directions, enhancement of IL-10 synthesis, and suppression of TNF formation by cAMP argues against a nonspecific effect of (Rp)-cAMPS. Concentrations between 200 and 1000 μ M (Rp)-cAMPS [35, 36], (Sp)-cAMPS [37], and similar agents like dibutyryl-cAMP [38] have been used effectively in different cell systems.

Toxic effects were excluded because measurement of TNF formation revealed the opposite effect, namely a reversal of rolipram-suppressed TNF production (data not shown [39]). Conversely, the protein kinase A activator (Sp)-cAMPS when added to LPS-stimulated PBMC mimicked the effects of rolipram and cicaprost in respect to both cytokines, IL-10 and TNF.

It has been suggested that endogenous IL-10 mediates cAMP-induced suppression of TNF synthesis [25]. The time pattern of TNF production and IL-10 formation in our study shows that in the initial phase, up to 4 h after LPS stimulation, rolipram suppresses TNF synthesis in the absence of effective IL-10 concentrations. This indicates IL-10-independent TNF-suppressing activity of rolipram, at least in the early stimulation period.

On the other hand, it has been demonstrated that endogenously produced TNF contributes to LPS-stimulated IL-10 synthesis [26]. Remarkably, the measurement of both cytokines in our experiments reveals that LPS-stimulated IL-10 production increases in the presence of cAMP-elevating agents despite a marked suppression of TNF formation. This demonstrates that the direct enhancing effect of cAMP on IL-10 synthesis is so pronounced that it overrides its indirect effect on IL-10 synthesis via endogenous TNF (which would lead to decreased IL-10 synthesis).

Elevated TNF concentrations in plasma or body fluids have been described for several diseases, such as rheumatoid arthritis, Crohn's disease, septic shock, and cerebral malaria. However, only recently, clear-cut evidence has been brought forward that TNF exerts a necessary role in some of these diseases. Three double-blind placebo-controlled studies with anti-TNF antibodies have shown a marked clinical and laboratory improvement in patients with rheumatoid arthritis [1], Jarisch-Herxheimer reaction [4, 40], and Crohn's disease [3]. However, blocking the TNF action by repeated administration of anti-TNF antibody, as shown for patients with rheumatoid arthritis [41], was accompanied by

antibody-related side effects. This underscores the need to investigate the regulatory mechanisms of TNF synthesis and the potential for pharmacological intervention.

Several animal studies underline the concept of TNF-mediated disease amenable to treatment with a specific type IV phosphodiesterase inhibitor. In a rat model of autoimmune encephalomyelitis marked TNF suppression and amelioration of disease was achieved by rolipram [42]. This was confirmed for experimental encephalomyelitis in non-human primates (marmosets) [43]. Turner et al. demonstrated suppression in TNF synthesis and enhanced survival by rolipram treatment in a rat model of acute respiratory distress syndrome [44]. The mechanism resulting in a protective effect in these studies is probably not limited to suppression of TNF synthesis but may comprise other effects such as marked increase of IL-10 synthesis.

Studies on IL-10 in chronic inflammatory disease such as chronic inflammatory bowel disease ascribe a protective role to IL-10. In IL-10-deficient mice chronic enterocolitis was observed [45]. Furthermore, preliminary results show marked anti-inflammatory capacities of systemically administered IL-10 in patients with Crohn's disease [20]. One might speculate that the positive effect of both strategies, application of anti-TNF antibodies or recombinant IL-10, could be exerted in vivo by cAMP-elevating agents such as rolipram and cicaprost.

Taken together, these results contribute to the elucidation of the mechanism underlying the anti-inflammatory activities of cAMP-elevating agents and add another argument to investigate these potent agents in animal models of TNF-mediated disease.

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EDITORIAL REVIEW

The role of cAMP regulation in controlling inflammation

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In 1958, Sutherland and Rall identified adenosine 3', 5'-monophosphate (cAMP) as an intracellular second messenger of hepatic glycogenolysis [1]. Subsequently, cAMP was shown to act as second messenger for a variety of hormones, inflammatory mediators and cytokines, and has been shown to modulate models of immune and non-immune inflammation *in vivo* and a variety of cellular processes *in vitro*. Indeed, the current paper by Ottone et al. is typical of research in this area. The authors show that in a population of adherent neutrophils, the oxidative burst induced by exposure to granulocyte-monocyte colony-stimulating factor is reduced by agents that elevate cAMP [2]. They speculate that therapeutic elevation of cAMP will result in reduced oxidative damage to tissues in neutrophil-dominated inflammatory reactions.

Production of cAMP in leucocytes is stimulated by β -adrenergic catecholamines, histamine and the E series prostaglandins by a receptor-coupled activation of adenylate cyclase, an enzyme which catalyses the conversion of adenosine triphosphate to cAMP [3]. Rises in intracellular cAMP are usually transient, cAMP being rapidly broken down by phosphodiesterases (PDEs) to 5'AMP. A role for cAMP in a particular cell function can be inferred from the use of agents that activate adenylate cyclase (receptor-coupled activation or direct activation with agents such as cholera toxin [4] or forskolin [5]), duplication of the cell response with a hydrophobic (i.e. membrane-permeable) analogue of cAMP (e.g. dibutyryl cAMP), inhibition of PDEs with methylxanthines (e.g. theophylline [6]) or isoenzyme-specific agents (see below) and by assessing the effects of these various treatments on intracellular cAMP levels.

At an inflammatory site, mast cells are stimulated to degranulate, causing release of vasoactive and other inflammatory mediators. Circulating leucocytes adhere to vascular endothelium and accumulate at the inflamed site under the direction of chemotactic factors. Phagocytic stimuli cause release of lysosomal enzymes and reactive oxygen species (ROS) from neutrophils, eosinophils and macrophages. Antigen recognition causes proliferation and differentiation of lymphocyte subsets. *In vitro* work has suggested that following cell stimulation, agents that elevate cAMP reduce: immunological release of histamine and leukotrienes from mast cells [7], monocyte [8] and neutrophil [9,10] locomotion, release of

lysosomal enzymes [11], ROS [12], platelet-activating factor [13] and leukotriene B₄ [14] from neutrophils, release of ROS from eosinophils [15], release of cytokines [16,17] and nitric oxide [18] from macrophages, proliferation of lymphocytes [19] and effector functions of cytotoxic T lymphocytes [20]. However, it is important to realize that the ability of cAMP elevating agents to suppress cell functions is not uniform but depends on the initial stimulus. In *in vivo* models of inflammation it has been shown that in different types of experimental pleurisy (carrageenan [21], pyrophosphate [22], Arthus [23] and delayed hypersensitivity [24]) cAMP levels vary during the reactions, low levels being observed as the reactions proceed and normal or higher levels being observed as the reactions subside [25]. The experimental data therefore suggest that cAMP is part of an endogenous mechanism for down-regulating the inflammatory response and preventing the beneficial effects of acute inflammation from progressing to chronic inflammation and its associated tissue destruction. This view is supported by the clinical finding that leucocytes from atopic individuals appear to have higher than normal PDE activity [26].

The targeting of a single mediator or group of mediators for treatment of inflammation has the drawback that other mediators could partially compensate for the loss, thereby limiting the efficacy of the treatment. Therapeutic elevation of cAMP to treat inflammatory disorders is attractive because a whole host of inflammatory cell functions can in theory be inhibited. In addition, *in vitro* work suggests that a synergy exists between activators of adenylate cyclase and PDE inhibitors in elevating cAMP. If this is true also *in vivo* then the production of agents such as prostaglandin E₂ (PGE₂) at a site of inflammation should ensure that the inflamed tissue is more responsive to PDE inhibitors than non-inflamed tissues.

Interestingly, and somewhat paradoxically, many non-steroidal anti-inflammatory drugs (NSAIDs) appear to elevate cAMP [27] despite blocking the synthesis of PGE₂ which stimulates adenylate cyclase. The reasons for this are unclear, but blockade of cyclooxygenase by these drugs could lead to an accumulation of its substrate, arachidonic acid, which has been shown to have second messenger properties [28]. It is clear that signal transduction pathways do not work in isolation, instead they interact to modulate cell responses [29]. Arachidonic acid appears to be able to elevate cAMP [28], which may explain the effects of NSAIDs on cAMP levels.

Theophylline has been used in the treatment of asthma for many years, and appears to be effective due to a combination of anti-inflammatory and bronchodilatory activities. However,

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theophylline is associated with side-effects in the gut, cardiovascular system and the central nervous system, and these side-effects seem to be mainly due to inappropriate inhibition of PDEs in these tissues and additional actions such as antagonism of adenosine receptors and stimulation of catecholamine release [30]. The future of PDE inhibitors as therapeutics therefore looked bleak until the realization that hydrolysis of cAMP (and cGMP) is not dependent on a single enzyme but on a range of isoenzymes which differ in their tissue distributions.

Seven families of PDEs (types I–VII) are currently recognized based on protein sequence and cDNA analysis. These enzymes differ in substrate selectivity, sensitivity to calcium/calmodulin, allosteric regulation by cGMP, sensitivity to phosphorylation and distribution both in tissues and subcellular compartments [30–33]. Each family can contain subfamilies, and further diversification may arise from genes that can give rise to two or more alternatively spliced RNAs. Tissues may express more than one family of PDEs, but in inflammatory cells (with the exception of lymphocytes) it seems to be members of the PDE IV family that are dominant. Lymphocytes appear to have both PDE III and PDE IV enzymes; whether particular isoenzymes are confined to particular subsets of lymphocytes is not known. PDE IV enzymes are cAMP-specific, are calcium/calmodulin-independent, and are not regulated by cGMP. In addition to inflammatory cells, PDE IV enzymes are found in smooth muscle, brain, liver, heart and kidney. PDE IV inhibitors should lack activities other than PDE inhibition and be more tissue selective than theophylline. However, the distribution of PDE IV enzymes suggests that major side-effects could still be a problem. Indeed, PDE IV inhibitors are being developed as anti-depressants; what effect these drugs would have on unaffected individuals is not known. As subfamilies of PDE IV are investigated, isoenzymes that are truly specific to inflammatory cells may become apparent which will prove more effective targets.

Interest in PDE inhibitors has increased enormously since the discovery of isoenzymes with differing tissue distributions. The potential therapeutic advantages of PDE IV inhibitors in the treatment of inflammatory diseases are clear. However, it is only as data become available from clinical trials that we will see whether these compounds live up to their potential.

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INCREASED CYCLIC AMP-PHOSPHODIESTERASE ACTIVITY DURING INFLAMMATION AND ITS INHIBITION BY ANTI-INFLAMMATORY DRUGS *

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Cyclic AMP-phosphodiesterase (cAMP-PDE) activity was determined in edematous and granulomatous tissues in carrageenin hind paw edema and cotton pellet granuloma of rats. In the time course study, maximum cAMP-PDE activity was observed in edematous tissue at 3-6 h after carrageenin injection and in granulomatous tissue from the 7th day onwards after cotton pellet implantation. Pretreatment with anti-inflammatory drugs inhibited significantly the increase in cAMP-PDE activity in edematous and granulomatous tissues. It was also observed that the increase in cAMP-PDE activity in edematous tissue paralleled the increase in migrated cells (leukocytes). Therefore, it is possible that the inhibition of cAMP-PDE activity by anti-inflammatory drugs may be the result of inhibition of cellular infiltration into the inflammatory site. It is suggested that phosphodiesterase may be an additional biochemical parameter for assessing cellular events of inflammation and consequently useful for the evaluation of anti-inflammatory drugs.

Inflammation

Cyclic AMP-phosphodiesterase

Anti-inflammatory drugs

1. Introduction

Intracellular cyclic nucleotide levels in biological systems can be controlled through regulation of either synthesis via the respective cyclases or of degradation via phosphodiesterase (PDE) (Beavo et al., 1970). Cyclic AMP has been proposed as a modulator of the physiological responses of the effector cells of inflammation (Kaliner and Austin, 1984). In addition, it has also been shown that cyclic adenosine monophosphate alone or in combination with theophylline suppressed the acute and the chronic type of inflammation in rats (Ichikawa et al., 1972). Furthermore, lower levels of cyclic AMP have been reported in gingival tissue inflammation and considered to be one of the mediators of the physiological changes seen in

gingival tissue during inflammation (Grower and Chandler, 1979).

In the light of the above information, it was thought worthwhile to study and analyse the changes in phosphodiesterase activity in experimentally induced inflammation and to look for a relationship between the pharmacological inhibition of cAMP-PDE and the inhibition of inflammation.

2. Materials and methods

2.1. Acute inflammation

Male albino rats of Wistar strain, weighing 130-150 g were used. The rats were housed in an air-conditioned room maintained between 20 and 22°C and illuminated from 6 a.m. to 6 p.m. each day. The rats were given pellet food and water ad libitum.

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Carrageenin edema was produced acutely by injecting the phlogistic agent, Carrageenin (Marine Colloids, Inc., Springfield, NJ 07081, U.S.A.) into the plantar region of the hind paws of rats according to the method of Winter et al. (1962). The rats were sacrificed at different time intervals, i.e., 2, 4, 6, 12, 24 and 48 h after carrageenin injection. cAMP-PDE activity was determined in edematous tissue by removing edema tissue along with exudate from the whole paw leaving the cartilage.

In order to see the effect of non-steroidal anti-inflammatory drugs on the cyclic adenosine monophosphate-dependent phosphodiesterase (cAMP-PDE) activity in edematous tissue, drugs (indomethacin, phenylbutazone, aspirin and ibuprofen) were administered at different doses orally to separate groups of rats 1 h prior to carrageenin injection. The control group received vehicle instead of drugs. The animals were sacrificed 6 h after carrageenin injection.

In order to see the effect of drugs alone on the activity of normal paw cAMP-PDE, the animals were treated with drugs at the same dose level as used in carrageenin edema, sacrificed 7 h after drug treatment and whole paw tissue then removed leaving cartilage. This procedure could help to distinguish the effect of drugs on inflamed and non-inflamed tissues. The paw volume of rats was measured on a plethysmographic apparatus prior to and 6 h after drug treatment. In these experiments the 50% inhibitory dose (ID_{50}) for cAMP-PDE in edematous tissue as well as the 50% effective dose (ED_{50}) for edema inhibition was calculated for each drug.

2.2. Cellular response and the effect of anti-inflammatory drugs

Groups of rats injected with carrageenin were sacrificed at different time intervals (1, 3, 6, 24 h) and the hind paws of the rats were carefully dissected and the exudate from the paw was removed. The volume of exudate was recorded using siliconised pasteur pipettes. Differential white cell counts were made on the stained smears using counting chambers. In another set of experiments the rats with carrageenin edema were pretreated with anti-inflammatory drugs at the highest effec-

tive dosage and sacrificed 6 h after carrageenin injection. The same procedure as above was repeated for determining exudate cell counts.

2.3. Chronic inflammation

Cotton pellet granuloma was produced in rats by the method of Winter and Portar (1957) with slight modifications. Drugs (phenylbutazone (30 mg/kg), indomethacin (1 mg/kg), aspirin (100 mg/kg) and ibuprofen (0.5 mg/kg)) were administered orally daily to rats in a volume of 1.0 ml/100 g body weight for a period of 7 days. On the 8th day rats were sacrificed and granulomatous tissues separated for determination of cAMP-PDE activity. Various other doses of these drugs were also used for each drug to calculate the 50% inhibitory dose (ID_{50}) for cAMP-PDE in granulomatous tissues and as well as the 50% effective dose (ED_{50}) for the inhibition of granuloma tissue formation. In order to investigate time course changes in granuloma formation, rats from these groups were sacrificed on the 3rd, 5th, 7th, 10th and 15th days and granulomatous tissue was separated for the determination of cAMP-PDE activity. Normal rats were sacrificed on similar days for the determination of skeletal muscle cAMP-PDE activity. Granulomatous cAMP-PDE activity was compared with normal skeletal muscle (from the ventral region) cAMP-PDE activity.

2.4. Ulcer formation

In order to see whether the doses of drugs employed in the present study (both in the carrageenin and cotton pellet granuloma group) would cause ulceration of the mucosa, the stomach was removed, washed with saline and cut open along the lesser curvature for microscopic examination of the gastric mucosa.

2.5. Biochemical estimation

Edema tissue along with exudate was removed from the hind paws by careful dissection. Granulomatous tissue along with a small amount of exudate was removed similarly from the cotton pellet granuloma. The whole operation was carried

out at 0-4°C and the homogenates of edematous, granulomatous, skeletal muscle or normal tissues were prepared by homogenizing in Tris-HCl buffer pH 8.6 using a motor driven Potter Elvehjem glass homogenizer.

cAMP-PDe enzyme hydrolyses cAMP completely to adenosine-5-monophosphate (Sutherland and Rall, 1958; Robinson et al., 1971). The 5'-AMP is then broken down enzymatically to adenosine and inorganic phosphate by 5'-nucleotidase from snake venom (Butcher and Sutherland, 1962). Inorganic phosphate can then be estimated spectrophotometrically. The PDE present in snake venom is shown to hydrolyse cAMP slowly and in 30 min incubation period the breakdown of cAMP to 5'-AMP is negligible. The whole procedure was carried out in an icebath. An assay mixture containing 2.5 mmol Tris-HCl buffer pH 8.6, 2.5 mol mg Cl₂, 10 mmol (NH₄)₂SO₄, 0.75 mmol adenosine-3',5'-cyclic monophosphate (sodium salt, Sigma Chemicals, U.S.A.) and 0.1 ml of 10% (w/v) tissue homogenate (source of cAMP phosphodiesterase) in a final volume of 0.6 ml was incubated in a water bath at 37°C for 30 min. Each test sample was accompanied by a trichloroacetic acid (TCA)-treated homogenate as control. The reaction was stopped by immersing the tube in boiling water bath for 2 min. The tubes were cooled, kept in an ice-bath and 0.5 ml of cobra Naja Venom (Haffkine Institute, Bombay, India) 10 mg/ml prepared in 0.5 M Tris-HCl buffer pH 8.6 was added to all tubes. The tubes were reincubated at 37°C for 30 min. After 30 min of incubation the reaction was stopped instantly by adding 1.0 ml of ice-cold 10% TCA. The volume of

the reaction mixture in each tube was made up to final volume of 2 ml with 10% TCA.

The reaction mixture then contained an inorganic phosphate released from the enzymatic breakdown of cAMP and which could be estimated spectrophotometrically. Proper controls without venom were run in parallel to exclude inorganic phosphate from the venom source. The estimation of inorganic phosphate was carried out by the method of Gomori (1942) using metol as a reducing agent. The protein content of the homogenate was assayed according to the method of Lowry et al. (1951).

2.6. Statistics

Results were expressed as means \pm S.E.M. and the statistical significance of the difference between means was calculated by Students t-test.

3. Results

cAMP-PDE activity was elevated in both edematous and granulomatous tissues (table 1-4).

The time course study with carrageenin edema and cotton pellet granuloma (tables 1 and 2) demonstrated that the increase in cAMP-PDE activity in these tissues was time-dependent. The maximum cAMP-PDE activity was observed at 6 h after carrageenin injection and on the 7th day after cotton pellet implantation in carrageenin edema and cotton pellet granuloma respectively. Incidentally this coincided with the time course of the response of carrageenin edema and cotton

TABLE 1

cAMP-phosphodiesterase activity during carrageenin edema in rats - time course study. 10 rats per group were used for both normal and inflammation experiments at each interval.

Time interval:	Specific cAMP-phosphodiesterase activity in nmol inorganic phosphorus released per mg enzyme protein in 30 min (mean \pm S.E.M.)					
	2 h	4 h	6 h	12 h	24 h	48 h
Normal paw tissue	5.7 \pm 0.8	5.2 \pm 0.5	5.8 \pm 0.9	6.0 \pm 0.7	5.2 \pm 0.1	5.8 \pm 0.4
Edematous tissues	7.5 \pm 0.5	9.7 \pm 0.1	15.6 \pm 0.4	9.4 \pm 0.2	7.1 \pm 0.8	6.2 \pm 0.4

TABLE 2

cAMP-phosphodiesterase activity during cotton pellet granuloma formation in rats - time course study. 10 rats per group were used for both skeletal muscle and granuloma experiments at each interval. Granulomatous cAMP-PDE was compared with skeletal muscle cAMP-PDE.

Time interval:	Specific cAMP-phosphodiesterase activity in nmol inorganic phosphorus released per mg enzyme protein in 30 min (mean \pm S.E.M.)				
	3rd day	5th day	7th day	10th day	15th day
Skeletal muscle tissue	4.9 \pm 0.8	5.0 \pm 0.6	4.7 \pm 0.8	4.9 \pm 0.4	5.0 \pm 0.9
Granulomatous muscle	12.0 \pm 0.8	21.0 \pm 0.5	36.0 \pm 4.0	32.0 \pm 5.0	30.0 \pm 3.8

pellet granuloma formation (unpublished observation).

Pretreatment with non-steroidal anti-inflammatory drugs prevented the increase in cAMP-PDE activity in edematous as well as granulomatous tissue (tables 3 and 4). However, pretreatment with non-steroidal anti-inflammatory drugs,

whether acutely or chronically, failed to inhibit basal cAMP-PDE activity in normal paw tissue or skeletal muscle tissue (tables 3 and 4). Moreover, neither of the drugs at these dose levels produced frank ulceration in carrageenin edema and in cotton pellet granuloma rats. All the drugs employed in carrageenin edema are known to elicit some hyperaemia and small foci haemorrhage in rats. The doses of non-steroidal anti-inflammatory drugs required for 50% inhibition of edema and granuloma formation and 50% inhibition of cAMP-PDE activity in edematous and granulomatous tissues were different for each drug. However, the potency of non-steroidal drugs in both types of inflammation for inhibiting the activity of

TABLE 3

Acute (single dose) effect of anti-inflammatory drugs on the cAMP-PDE activity of normal and edematous paw tissues.
 * Compared with vehicle-treated normal paw tissue. ** Compared with vehicle-treated edematous group. N.S., not significant.

Drug treatment and dose (p.o.)	No. rats	Specific cAMP-PDE activity in nmol of inorganic phosphorus released per mg enzyme protein in 30 min (mean \pm S.E.M.)
Normal		
vehicle 1 ml/100 g	10	5.8 \pm 0.9
aspirin 250 mg/kg	10	5.8 \pm 0.42
phenylbutazone 100 mg/kg	10	5.9 \pm 1.2 *
indomethacin 10 mg/kg	10	5.0 \pm 0.6 *
ibuprofen 8 mg/kg	10	5.2 \pm 0.3 *
Edematous		
vehicle 1 ml/100 g	10	16.6 \pm 1.2 *
aspirin 250 mg/kg	10	8.7 \pm 1.6 **
phenylbutazone 100 mg/kg	10	7.8 \pm 1.0 **
		P < 0.001
indomethacin 10 mg/kg	10	6.0 \pm 0.9 **
		P < 0.001
ibuprofen 8 mg/kg	10	7.4 \pm 1.2 **
		P < 0.001

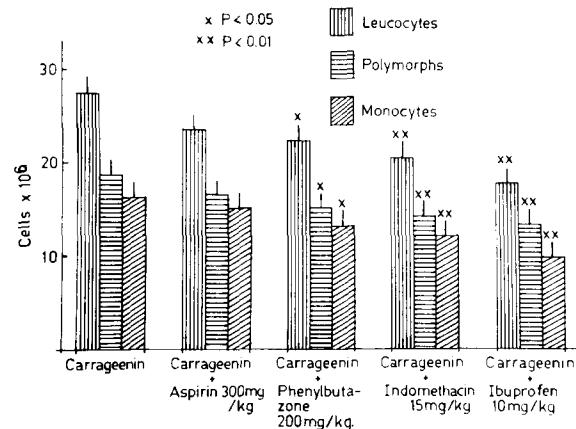


Fig. 1. Cellular response in carrageenin edema and effect of anti-inflammatory drugs on this response. Carrageenin edema rats were pretreated with anti-inflammatory drugs and sacrificed 6 h after carrageenin injection. Cell counts were made from edema exudate on the stained smears using counting chambers. Vertical lines show cell counts (mean \pm S.E.M.).

TABLE 4

Chronic (7 days treatment) effect of anti-inflammatory drugs on granulomatous cAMP-PDE activity in cotton pellet granuloma.
 * Compared with vehicle-treated skeletal muscle. ** Compared with vehicle-treated granulomatous group. N.S., not significant.

Drug treatment and dose (p.o.)	No. rats	Specific cAMP-PDE activity nmol inorganic phosphorus released per mg enzyme protein in 30 min	
		Granulomatous tissue (mean \pm S.E.M.)	Skeletal muscle tissue (mean \pm S.E.M.)
Vehicle 1 ml/100 g	10	33.0 \pm 1.2 * P < 0.001	4.8 \pm 0.9
Aspirin 100 mg/kg	10	22.0 \pm 1.4 ** P < 0.001	4.8 \pm 0.6 *
Phenylbutazone 30 mg/kg	10	18.0 \pm 0.78 ** P < 0.001	4.6 \pm 0.8 *
Indomethacin 1 mg/kg	10	10.0 \pm 0.48 ** P < 0.001	5.0 \pm 0.6 *
Ibuprofen 0.5 mg/kg	10	15.0 \pm 0.7 ** P < 0.001	4.9 \pm 0.8 N.S.

edematous or granulomatous cAMP-PDE remained the same and was of the order: ibuprofen > indomethacin > phenylbutazone > aspirin (table 5).

The cell counts, viz. leukocytes, polymorphs and monocytes, were increased and reached a maximum at 6 h and a minimum at 24 h after carrageenin injection (data not shown). Treatment with aspirin, phenylbutazone, indomethacin and ibuprofen decreased cell counts significantly (fig. 1). The inhibition of cell counts by these drugs was

in the following order of potency: ibuprofen > indomethacin > phenylbutazone > aspirin (fig. 1).

4. Discussion

This study demonstrated that both acute and chronic inflammation due to a local irritant was associated with an increase in the cAMP-PDE content of the edematous and the granulomatous tissue. The significant increase in cAMP-PDE

TABLE 5

ID₅₀ for cAMP phosphodiesterase activity in edematous and granulomatous tissues and ED₅₀ for anti-inflammatory activity of nonsteroidal anti-inflammatory drugs. Carrageenin edema was produced by the method of Winter et al. (1962) and different drugs at various doses were administered orally 1 h prior to carrageenin injection. Paw volumes were measured before and 6 h after carrageenin injection with a plethysmographic apparatus and ED₅₀ was calculated after determining edema inhibition at each dose level. These rats were sacrificed and cAMP-PDE was determined. Cotton pellet granuloma was produced by the method of Winter and Portar (1957) and drugs were given orally daily for 7 days. Rats were sacrificed on the 8th day and granulomatous tissues were separated and cAMP-PDE was assayed.

Drugs	ID ₅₀ for cAMP-PDE activity (mg/kg)		ED ₅₀ for anti-inflammatory activity (mg/kg)	
	Carrageenin edematous tissue (at 6 h)	Granulomatous tissue (on 8th day)	Carrageenin edema	Cotton pellet granuloma
Aspirin	300	200	200	250
Phenylbutazone	100	30	120	50
Indomethacin	8.0	0.75	8	3.0
Ibuprofen	7.0	0.6	5	2.5

observed in edematous and in granulomatous tissue is difficult to understand, however it is possible that cellular infiltration into the inflamed site (fig. 1) may be a triggering factor for the increase in cAMP-PDE seen during inflammation. This is also supported by the further observation that anti-inflammatory drugs selectively inhibited inflamed tissue cAMP-PDE and not cAMP-PDE activity in normal tissue. The increase in cAMP-PDE will reduce cAMP content of inflamed tissue and hasten the inflammatory process because cyclic AMP has been proposed as a modulator of the physiological responses of the effector cells of inflammation (Kaliner and Austin, 1974; Leh-meyer and Johnston, 1978). Moreover, it has been shown that the increased intracellular concentration of cAMP inhibited the leukotactic response of neutrophils to bacterial chemotactic factors and that a decrease in cellular cAMP levels enhanced the leukotactic response (Hill et al., 1975). Cyclic AMP has also been reported to inhibit the release of histamine from leukocytes of patients with a ragweed hayfever (Bourne et al., 1974) and in isolated rat peritoneal mast cells (Kaliner and Austin, 1974).

Unfortunately all these experiments have been carried out with in vitro systems and their real applicability to natural inflammation is still questionable. However, Deporter (1977) has shown experimentally in an in vivo system that there was a close relationship between endogenous leukocyte cAMP levels and the progression of acute inflammation. Various phosphodiesterase inhibitors were used in these experiments. Parnham et al. (1978) observed decreased cAMP levels in rat hind paw during the development of adjuvant arthritis and suggested that this change in cAMP level indicated the infiltration of activated leukocytes into the inflamed joint. Furthermore, Grower and Chandler (1979) have observed low levels of cAMP in gingival tissue during inflammation and proposed that cAMP may be one of the subcellular mediators of inflammation in the periodontium.

On the other hand, Willoughby et al. (1975) reported that in other models of acute inflammation there was an immediate increase in cAMP level in the exudate cells and thereafter a sharp decline to a very low cAMP level at the hour of

peak inflammation. These different results may be attributed to different types of local irritant (calcium pyrophosphate crystal) being used in the production of inflammation and also to the different mode of action of this irritant in eliciting inflammation.

To summarise and conclude, it was observed that anti-inflammatory drugs inhibited significantly the increased cAMP-PDE activity in edematous and granulomatous tissues. It has been postulated that anti-inflammatory drugs (e.g. indomethacin and other non-steroidal anti-inflammatory drugs) interact with the adenylate cyclase system and inhibit PDE thereby elevating the intracellular concentration of cyclic AMP (Weiss and Hait, 1977). Deporter et al. (1979) have found increased cAMP levels in leukocytes after indomethacin treatment. Furthermore, studies on chicken epiphyseal and particular cartilage (Newcombe et al., 1974) and human synoviocytes (Ciosek et al., 1974) have also shown that indomethacin inhibited both prostaglandin and cAMP-PDE. The results from this study and from other work discussed in this paper allow me to propose that cAMP may be one of the subcellular mediators of inflammation. It seems that increased activity of phosphodiesterase(s) is associated with increased inflammation. There is good agreement between anti-inflammatory activity and biochemical effects (cAMP-PDE inhibition) of anti-inflammatory drugs. In addition, PDE may serve as an additional biochemical parameter for assessing cellular events of inflammation and consequently be useful for evaluation of anti-inflammatory drugs. It is proposed that the decreased cAMP level in the inflamed tissue may account for the increased inflammatory filtrate which may lead to collagen breakdown of the tissues. Though this hypothesis appears to be an attractive one, at the present time it is based purely on circumstantial evidence.

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Regulation of T-cell apoptosis in inflammatory bowel disease: to die or not to die, that is the mucosal question

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T-cell resistance against apoptosis contributes to inappropriate T-cell accumulation and the perpetuation of chronic mucosal inflammation in inflammatory bowel diseases (IBDs). Anti-interleukin-12 (IL-12) and anti-IL-6 receptor antibodies suppress colitis activity by the induction of T-cell apoptosis. These findings have important implications for the design of effective treatment regimens in IBD.

Studies in experimental models of mucosal inflammation have led to major new insights into the abnormalities present in their counterpart human diseases, the inflammatory bowel diseases (IBDs) as well as exciting new approaches to the therapy of these diseases^{1–3}. Two such approaches, the administration of antibodies against interleukin 12 (IL-12) and the IL-6 receptor (IL-6R) have been shown to be dramatically effective in both the prevention and treatment of experimental mucosal inflammation^{4–7}. This initially appeared to be due to the ability of these antibodies to neutralize key cytokines responsible for the inflammation, either during its inductive phase (anti-IL-12) or its effector phase (anti-IL-6R). However, recent work has disclosed that the mechanisms underlying the therapeutic effects of these antibodies are more profound^{8,9}: both affect the survival of CD4⁺ T cells mediating the inflammation, such that the main action of the antibodies is on the cells inducing or producing the cytokines rather than the cytokines themselves. These findings have important implications for the design of more-effective cytokine-based treatments for IBDs and other inflammatory states that have a similar pathogenesis.

Regulation of apoptosis of lamina propria T cells via active and passive mechanisms

Immune responses in the mucosa are frequently characterized by major expansions of antigen-specific T cells that have potent effector function^{1,8–10}.

Although this might be important for host defense, it might also lead to effector cell populations with substantial autoreactivity and the capacity to cause mucosal inflammation. To deal with this latter possibility, the mucosal immune system has evolved several strategies for the control of mucosal immune responses. Among these is the regulation of the programmed cell death (apoptosis) that either occurs via an active mechanism following T-cell receptor (TCR) stimulation (activation-induced cell death) or via a passive mechanism following lymphokine (for example, IL-2) withdrawal^{11,12}. The active mechanism involves death receptors such as Fas and tumor necrosis factor receptor (TNFR) and/or their ligands (FasL and TNF), whereas the passive mechanisms do not. To understand how these forms of apoptosis operate within the mucosal immune system at a molecular level, we must first describe the major signaling events occurring during death-receptor-mediated apoptosis and their interactions with apoptosis mechanisms mediated by mitochondria.

Death mediated by Fas–FasL

Death-receptor-induced apoptosis comprises signaling processes via Fas (CD95, APO-1), TNFR1, TRAIL-R1 and 2, and DR3 or DR6 (Refs 11,13). Upon triggering of Fas by FasL, the adaptor molecule FADD (Fas-associated death-domain-containing protein) and pro-caspase 8 are recruited to the Fas receptor, thereby forming a death-induced signaling complex (DISC) (Fig. 1)^{11,14,15}. Recruitment of pro-caspase 8 leads to its autoproteolytic activation and the release of active caspase 8 (FLICE) into the cytosol, where it can trigger two signaling pathways. The first pathway of Fas-mediated apoptosis is activated by low amounts of caspase 8 and involves the cleavage of the pro-apoptotic BID molecule, followed by the release of cytochrome *c* from mitochondria into the cytoplasm, and subsequent cell death¹⁴. The second pathway involves large amounts of caspase 8 that bypass mitochondria and activate other caspases such as caspase 3 (Fig. 1). Because Bcl-2 and Bcl-x_L only inhibit apoptotic activities of mitochondria, the first but not the second pathway of active apoptosis is inhibited by these anti-apoptotic proteins¹³. Passive forms of apoptosis, on the other hand, involve activation of BID proteins that act exclusively via mitochondrial mechanisms; thus, passive apoptosis as caused by lymphokine withdrawal, is strongly inhibited by Bcl-2 and Bcl-x_L.

Apoptosis of lamina propria CD4⁺ T cells in the normal and inflamed gut

As most T cells in the lamina propria (LP) express memory cell markers such as CD45RO (Ref. 3), the activation-induced cell death might be very important to downregulate effector cell function and cytokine production in the gut. Interestingly, memory T cells are known to express Fas constitutively at high levels¹³. In the unstimulated state, (that is, prior to TCR stimulation), LP T cells exhibit increased susceptibility

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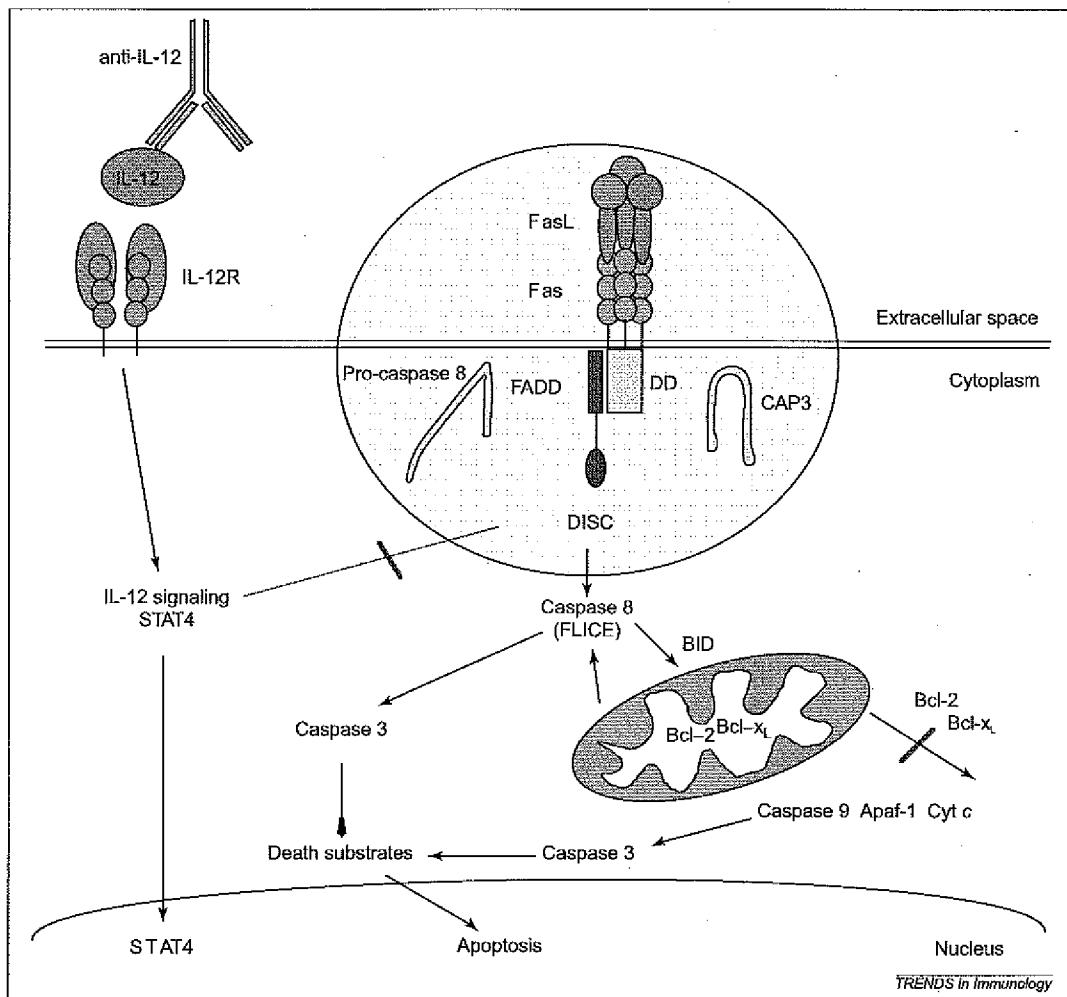


Fig. 1. Apoptosis of mucosal T cells via the Fas–FasL system and suppression of Fas-mediated apoptosis by IL-12. Normal LPT cells show an enhanced susceptibility to Fas-mediated cell death¹⁶. Upon activation of Fas, the proteins FADD, CAP3 and pro-caspase 8 are recruited to the death domain (DD) of the receptor, forming the DISC. DISC signalling events lead to activation of caspase 8 that, in turn, either directly activates caspase 3 or causes cytochrome *c* (Cyt *c*) release from mitochondria. Cyt *c* in the cytoplasm binds to Apaf-1, forming a complex that binds caspase 9 and causes its activation. Activated caspase 9 then activates other proteases, including caspase 3, and thus causes cleavage of various substrates that subsequently lead to the death of the cell¹¹. However, in chronic intestinal inflammation, increased production of IL-12 prevents Fas-mediated T-cell apoptosis, leading to T-cell accumulation in the gut. This mechanism of disease perpetuation can be successfully blocked by antibodies to IL-12 (Ref. 8). Abbreviations: DD, death domain; DISC, death induced signalling complex; FADD, Fas-associated death domain; FasL, Fas ligand; IL-12, interleukin 12.

to Fas-mediated apoptosis as compared with unstimulated peripheral-blood T cells; this is due to as yet unknown downstream changes in the Fas signalling pathway¹⁶. In addition, LP T cells exhibit increased spontaneous apoptosis compared with peripheral blood cells; this is probably due to a passive apoptotic mechanism associated with IL-2 withdrawal, because this apoptosis is diminished by addition of IL-2 and is induced by addition of anti-IL-2 antibodies. Similarly,

after stimulation of LP T cells with anti-CD2 plus anti-CD28 antibodies, apoptosis is further augmented compared with stimulated peripheral blood T cells¹⁶. This increased apoptosis is the result of activation-induced apoptosis involving death receptors, because it is observed when the cells are activated via the CD2 rather than the CD3 activation pathway, the CD2 pathway being a more effective means of activating LP T cells¹⁷. Furthermore, this apoptosis is extinguished by antibodies that block Fas, whereas addition of IL-2 or anti-IL-2 antibodies has little effect¹⁶.

In contrast to LP T cells from control patients, those from patients with IBD (Crohn's disease and ulcerative colitis) exhibit defective Fas-induced apoptosis upon stimulation via CD2 (Ref. 18). Although LP T cells in IBD express the same amount of Fas on their surface, they were less sensitive to Fas-mediated apoptosis than were control cells. However, resistance of LP T cells against apoptosis in Crohn's disease is not restricted to the Fas–FasL system, as recent data has shown that Crohn's disease T cells grow more rapidly in response to IL-2 than do control cells and are more resistant to IL-2-deprivation-induced apoptosis and apoptosis mediated by nitric oxide¹⁹. This broad resistance to

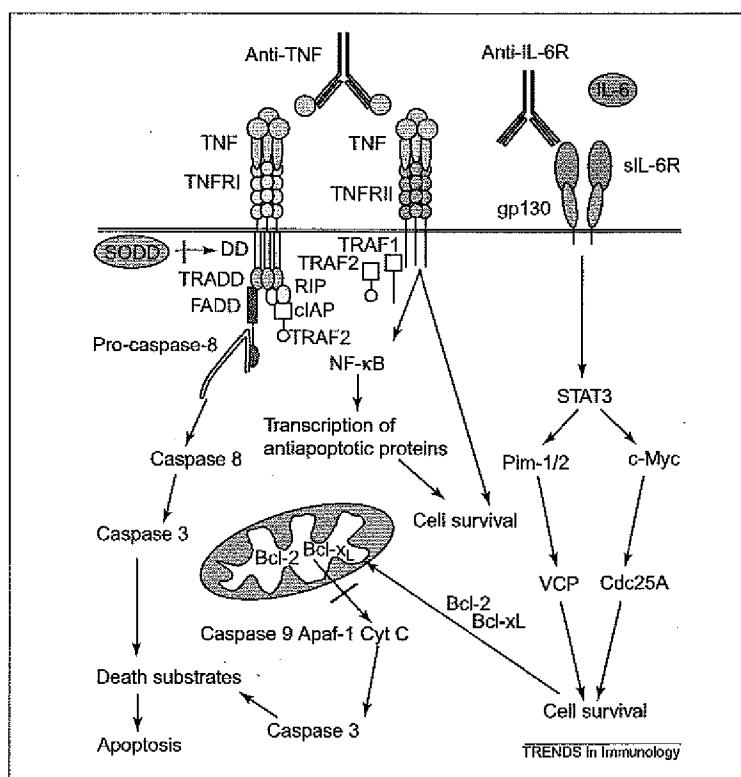


Fig. 2. Prevention of T-cell apoptosis in chronic intestinal inflammation by IL-6 trans-signaling and TNF signaling events. Chronic intestinal inflammation is associated with increased production of both TNF and IL-6 (Refs 6,35). IL-6 may bind to IL-6R⁺ LPT cells upon binding to its soluble receptor (sIL-6R) and the gp130 molecule. Signaling via gp130 results in activation of STAT3 and STAT1 target molecules such as Pim-1/2, c-myc and finally bcl-2 and bcl-x_L (Refs 23,25). This pathway causes enhanced cell survival and T-cell resistance against apoptosis in IBD (Ref. 6). Blockade of IL-6 trans-signaling causes amelioration of intestinal inflammation by suppression of T-cell resistance against apoptosis. Similarly to IL-6, TNF may induce enhanced cell survival by signaling via TNFR1/II and activation of NF- κ B, although activation of TNFR1 may also induce apoptosis via activation of caspase 8 (Ref. 13). However, recent data indicate that antibodies to TNF (which are clinically very effective in inducing rapid suppression of mucosal inflammation in IBD patients²⁷) appear to mediate their effects at least in part by rapid induction of T-cell apoptosis in the inflamed gut²⁸, suggesting an important function for TNF in regulating mucosal T cell survival in IBD patients. Abbreviations: Cyt c, cytochrome c; DD, death domain; DISC, death induced signaling complex; FADD, Fas-associated death domain; FasL, Fas ligand; IL-6, interleukin 6; IL-6R, interleukin 6 receptor; IL-12, interleukin-12; LP, lamina propria; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor.

apoptosis accords with the fact that T cells in inflamed tissue express increased levels of Bcl-2 (Ref. 6) and thus may be resistant to a range of apoptotic mechanisms that involve mitochondrial activity.

Overall, the picture that emerges is that, in the uninflamed gut, T cells manifest increased spontaneous or activation-induced apoptosis mediated by the Fas pathway that places a limit on the expansion of T cells following direct and bystander stimulation by specific antigen. By contrast, in inflamed tissue, T cells emerge that are resistant to apoptosis: thus, they exhibit prolonged survival and increased cytokine production that might, in turn, significantly aggravate the inflammation. Such resistance to apoptosis also appears to be important in various autoimmune diseases such as the autoimmune lymphoproliferative syndrome (ALPS). Although a severe defect in Fas-mediated apoptosis in ALPS can cause certain

non-gastrointestinal forms of autoimmunity²⁹, the resistance to apoptosis of LP T cells in IBD is not likely to be a primary cause of the disease; more likely, it is a secondary effect of inflammation that aggravates rather than causes the latter. However, the functional importance of T-cell resistance against apoptosis in the

Antibodies to pro-inflammatory cytokines appear to suppress chronic intestinal inflammation by the induction of T-cell apoptosis

inflammatory process is supported and significantly extended by recent studies in animal models of IBD showing that various antibodies to pro-inflammatory cytokines and their receptors, such as IL-12, TNF and IL-6R, appear to suppress chronic intestinal inflammation by the induction of T-cell apoptosis⁶⁻⁸.

IL-6 and IL-12 suppress apoptosis of mucosal T cells in chronic intestinal inflammation

IL-12 and apoptosis

Administration of a neutralizing anti-IL-12 antibody to mice with trinitrobenzene sulfonic acid (TNBS)-induced colitis, a T helper 1 (Th1)-mediated inflammation replete with cells producing large amounts of interferon- γ (IFN- γ), IL-12 and TNF- α , is followed after two days by the appearance of apoptotic (terminal deoxynucleotidyltransferase-mediated UTP end-labeling positive, TUNEL⁺) CD4⁺ cells at the site of inflammation in the colon⁸. This induction of T-cell apoptosis is followed by the rapid resolution of the inflammatory state, and might explain the dramatic suppression of intestinal inflammation in various Th1 models of colitis after treatment with anti-IL-12 antibodies^{4,5,21}. The anti-IL-12-induced apoptosis was a Fas-mediated phenomenon in that the cells being lost were preferentially Fas-bearing cells⁸. In addition, the therapeutic effect of the antibody was greatly diminished in MRL/lpr mice, which cannot mediate apoptosis via the Fas pathway, as well as in normal (SJL/J) mice administered Fas-Fc, an agent that blocks Fas-signaling via FasL, but does not itself signal via Fas (Ref. 8). These results strongly imply that activated Th1 cells producing inflammatory cytokines require the continued presence of IL-12 if they are to avoid a Fas-mediated death. The mechanism by which IL-12 counteracts the Fas pathway in such cells is not yet fully understood, although it is known that it does not involve the induction of the anti-apoptotic proteins, Bcl-2 or Bcl-x_L, and thus may act on mitochondria-independent DISC signaling events (Fig. 1).

IL-6 and apoptosis

Administration of anti-IL-6R antibodies to mice with various forms of experimental Th1-mediated colitides – including colitis associated with IL-10 deficiency, colitis

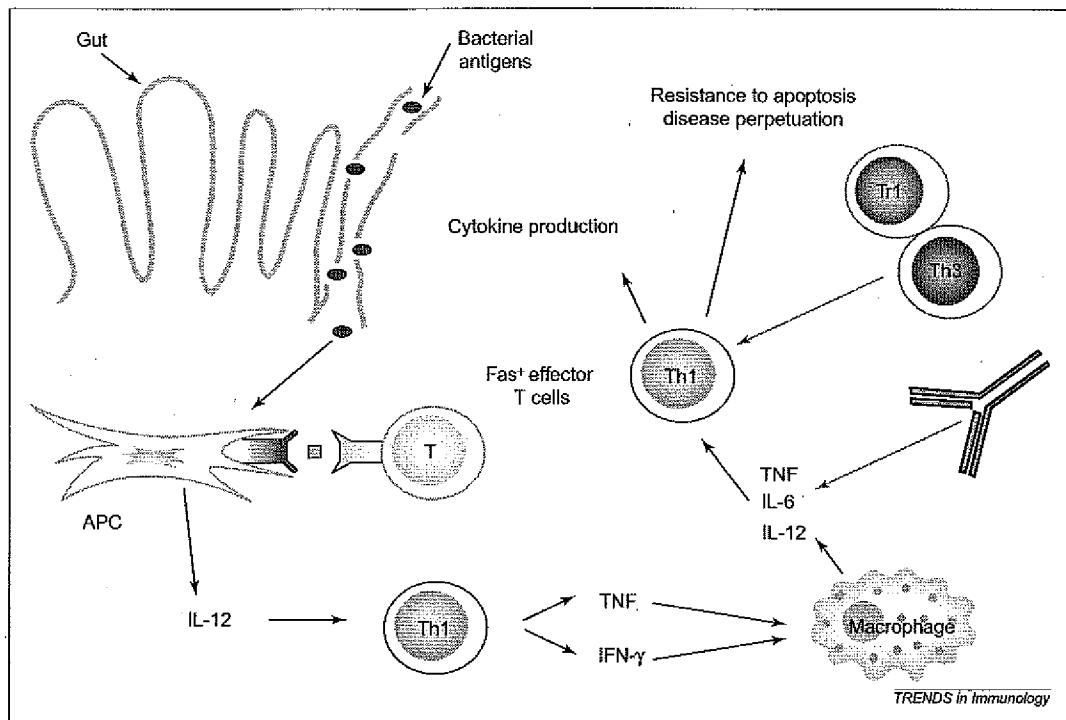


Fig. 3. T-cell resistance against apoptosis is a potential key factor in the pathogenesis of IBDs such as Crohn's disease. In this disease, bacterial antigens in the gut appear to induce T-cell activation and Th1 cell differentiation via IL-12 (Refs. 2–3, 36–37). Mediator substances from Th1 cells such as TNF and IFN- γ can activate macrophages to release TNF, IL-6 and IL-12, which in turn mediate T-cell resistance against apoptosis in the gut^{6,9}. This may result in prolonged cytokine production by Fas-expressing Th1 effector cells and, finally, tissue damage via activation of matrix metalloproteinases³⁸. This inflammatory cascade can be suppressed by suppressor cytokines released by regulatory Tr1 or Th3 cells^{32,33}. Alternatively, it can be arrested by antibodies such as anti-IL-12 and anti-IL-6R, which induce Th1-cell apoptosis. Thus, these antibodies alone, or in combination, provide an attractive new approach to the therapy of IBDs. Abbreviations: APC, antigen presenting cell; IBD, inflammatory bowel disease; IFN, interferon; IL-6, interleukin-6; IL-6R, interleukin-6 receptor; Th1, Thelper 1; TNF, tumor necrosis factor; Tr1, T regulatory cells 1.

occurring in severe combined immunodeficiency (SCID) mice upon transfer of naive (CD62L⁺, CD45Rb^{hi}) T cells, and TNBS-induced colitis – leads to a major reduction in the severity of colitis and the occurrence of apoptotic cells in the inflamed tissues^{6,7}. The mechanism underlying this anti-apoptotic effect proved to be quite unique in that it was mediated by complexes of soluble IL-6R (sIL-6R) and IL-6 that interact with gp130 on the membrane of CD4⁺ T cells and initiate an anti-apoptotic IL-6 signal. Support for such IL-6 *trans*-signaling comes from the fact that a gp130-Fc fusion protein that interacts with sIL-6R but not with membrane-bound IL-6R could also ameliorate various forms of colitis, presumably by blocking the interaction of the complex with membrane-bound IL-6R and thus the anti-apoptotic IL-6 signal⁶. In addition, it was shown that, while T cells from the LP of patients with Crohn's disease (a Th1-mediated inflammation of humans) lack IL-6R, they appear to have transduced an IL-6-specific signal *in vivo* in that they contain

activated STAT3. Furthermore, IL-6-sIL-6R complexes were found in the serum of Crohn's disease patients²², and treatment of Crohn's disease LP T cells with anti-IL-6R results in both the disappearance of cells containing activated STAT3 and increased cell apoptosis⁶. The anti-apoptotic effect mediated by IL-6 *trans*-signaling, in contrast to the anti-apoptotic effect mediated by IL-12, may involve Bcl-2 and Bcl-xL (Fig. 2)^{23,24}. Indeed, these proteins are elevated in LP cells from patients with Crohn's disease leading to an increase of the Bcl-2:Bax ratio¹⁹ and are known to be induced by STAT3 (Ref. 25). On the basis of these new data relating to IL-6R signaling, the abatement in apoptosis in the inflammatory milieu of the inflamed gastrointestinal tract can be attributed to the fact that in this milieu the cells are subject to IL-6R *trans*-signaling and the induction of anti-apoptotic proteins.

Concluding remarks

Apoptosis of LP T cells appears to be critical in the downregulation of the mucosal immune response and in the elimination of reactive clones. Whereas normal LP T cells exhibit an enhanced susceptibility to Fas-mediated apoptosis¹⁶, Crohn's disease is associated with a resistance of LP T cells against multiple apoptotic pathways^{6,18,19}. Such defective apoptosis may be a key factor for inappropriate T-cell accumulation and in the perpetuation of chronic mucosal inflammation in IBD (Fig. 3). Novel treatment modalities such as anti-IL-12 and anti-IL-6R antibodies appear to mediate their rapid beneficial effects on colitis activity at least in part by the suppression of T-cell resistance against apoptosis and the consecutive induction of mucosal T-cell death (Figs 1, 2). In addition, it should be noted that anti-TNF- α

antibodies that are currently used with considerable success in the treatment of Crohn's disease^{26,27} have also been suggested to act by inducing apoptosis of T cells²⁸.

Although treatment with anti-IL-12 or anti-IL-6R antibodies induces death of LP Th1 T cells, it is not clear at the moment whether such treatment would also induce cell death of regulatory Tr1 or Th3 cells; a phenomenon that would question the long-term beneficial effects of this therapeutic approach. However, several findings suggest that Th3 and Tr1 cells are not subject to anti-IL-12-mediated apoptosis. First, these cells are not Th1 cells and thus lack a fully competent IL-12 receptor; second, there is good evidence that TGF- β extinguishes IL-12R β 2 chain expression²⁹ so that Th3 cells would primarily downregulate IL-12R expression if any such existed; and third, administration of anti-IL-12 to mice has actually been shown to enhance TGF- β and IL-10 secretion³⁰.

The above findings on anti-IL-6R or anti-IL-12 treatment are relevant to human IBD, and to other human Th1- or Th2-mediated inflammatory conditions. Thus, in Th1-mediated inflammation such as Crohn's disease^{26,31}, the provision of an IL-12 inhibitor such as anti-IL-12 will not only result in resolution of disease by neutralization of the cytokines that initiate the Th1 pathway of inflammation, but also by the death of induced CD4 $^{+}$ Th1 cells. A similar effect can be achieved by the

provision of an IL-6 inhibitor such as anti-IL-6R since such therapy also results in both neutralization of cytokines and cell death. However, in this case, the therapy is probably not limited to Th1-mediated inflammation since it applies to end-stage CD4 $^{+}$ effector T cells arising from any T-cell differentiation pathway. Thus, anti-IL-6R antibodies might be used in ulcerative colitis, a form of inflammatory bowel disease that, in some aspects, is more like a Th2-mediated than a Th1-mediated inflammation³¹, in that neither IL-12 nor IFN- γ secretion is increased by isolated LP macrophages or T cells, respectively, and Th2 T cell-mediated mouse models of inflammation resemble ulcerative colitis pathologically^{2,32,33}.

The potential relevance of modulating T-cell resistance against apoptosis as a therapeutic target in IBD is underlined by the recent observation that drugs that facilitate Fas-mediated apoptosis can be used for suppression of T-cell-mediated autoimmune disease³⁴. However, it should also be noted that the T-cell death brought about by anti-IL-12 and anti-IL-6R antibodies in intestinal inflammation is likely to occur via a largely independent mechanism. This introduces the possibility of inducing death of inflammatory CD4 $^{+}$ T cells in independent and possibly synergistic ways. Thus, the future of therapy for many types of inflammation might lie in combined anti-cytokine therapy rather than therapy with a single agent alone.

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Mechanism of anti-D-mediated immune suppression – a paradox awaiting resolution?

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During pregnancy, women can be immunized by fetal red blood cells (RBCs) of an incompatible blood group. Subsequent transplacental passage of the antibodies can result in fetal morbidity or mortality due to RBC destruction. The administration of anti-D antibodies to D⁻ women after delivery of a D⁺ infant, and subsequent prevention of Rhesus (Rh) D haemolytic disease of the fetus and newborn, is the most successful clinical use of antibody-mediated immune suppression. The passive IgG anti-D might prevent immunization to D⁺ RBCs by an IgG Fc_γreceptor (Fc_γR)-dependent mechanism such as crosslinking the D-specific B-cell receptor and inhibitory Fc_γRllb. However, recent murine studies demonstrate that the suppressive effects of antibodies to heterologous RBCs can be Fc_γR-independent, suggesting other mechanisms might contribute.

Some antibody responses are not beneficial, for instance those to alloantigens present on human blood cells. Although transfusion of incompatible blood is avoided, transplacental passage of fetal blood to women during pregnancy or parturition often occurs. If this results in immunization, IgG antibodies will cross the placenta during that or a subsequent pregnancy and can mediate destruction of the fetal cells (Fig. 1a,b). The incidence of maternal antibody formation is determined by the immunogenicity of the antigen and by the frequency of blood group incompatibility. Of the red blood cell (RBC) antigens, Rhesus (Rh) D is responsible for the majority of cases of haemolytic disease of the fetus and newborn (HDFN; Rhesus disease), with Rh c and Kell causing disease less often. In addition, mothers can become immunized to platelet antigens, giving rise to neonatal alloimmune thrombocytopenia. Antibodies to the HPA-1a antigen are the most common cause of this disease.

It has been known for a century that passively transferred antibody can suppress the immune response to the corresponding cellular antigen¹. The most effective and widespread clinical application of antibody-mediated immune suppression (AMIS) is RhD prophylaxis, the routine administration of anti-D antibodies to D⁻ women after parturition to prevent immunization to fetal D⁺ RBCs. If untreated, the immunization rate would be approximately 15%. About 50 years ago, before the introduction of neonatal and obstetric treatments or prophylaxis, the death rate from HDFN was approximately 150 per 100 000 births, or 10% of perinatal deaths², and was a cause of great distress to some families with this blood group incompatibility.

In the 1960s, experimental studies in Liverpool and New York (using policemen and prisoners respectively) demonstrated that injection of passive anti-D prevented immunization to D⁺ RBCs (reviewed in Ref. 3). The rationale for administering anti-D to mediate Rh D immunosuppression was either to promote non-complement-mediated rapid clearance of the RBCs (and their destruction or deviation away from sites of antibody formation), or to suppress specifically the immune response to antigen using passive antibody (Fig. 1c). After successful clinical trials (Refs 3,4) routine anti-D prophylaxis was introduced over 30 years ago. In the UK, patients with fetomaternal haemorrhages (FMH) in excess of 4 ml are given a minimum dose of 100 µg (500 i.u.) intramuscularly with additional anti-D given at a ratio of 25 µg per ml fetal RBCs.

The Rh D polypeptide is an integral membrane protein expressed solely on human erythrocytes⁵. Approximately 16% of Caucasians are D⁻, owing to deletion of the *RHD* gene⁶ (human knockouts). Thus about 10% of all women receive anti-D postnatally due to the Rh haplotype frequency. Rh D prophylaxis has reduced the immunization rate by 90% and HDFN is now a rare disease. Antenatal prophylaxis could further reduce the sensitization rate, but to be effective this therapy requires a fourfold increase in anti-D immunoglobulin (Ig) and the supply of antibody (obtained by regular plasmapheresis of immunized donors who are periodically boosted with accredited D⁺ RBCs) is limited. Monoclonal anti-D could alleviate this shortfall. Anti-D antibodies are not elicited in mice, but fortunately, human monoclonal antibodies have been produced and several monoclonal anti-Ds have been tested in volunteers after *in vitro*

RELATED PROCEEDINGS APPENDIX

As stated in the *Related Appeals and Interferences* section above, there are no other appeals or interferences known to Appellants, the undersigned Appellants' representative, or the assignee to whom the inventors assigned their rights in the instant case, which would directly affect or be directly affected by, or have a bearing on the Board's decision in the instant appeal. As such this section is left blank.